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IN VITRO HEPATOPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF *DIPLAZIUM ESCULENTUM* (RETZ) SW. –A WILD FERN FROM WESTERN GHATS

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Diplazium esculentum, Hepatoprotective, anti-inflammatory, Chang cell line, COX-2 and 5-LOX activity

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ABSTRACT

Present study investigated hepatoprotective and anti-inflammatory activities of *Diplazium esculentum*. (Retz.) Sw, a fern from Wayanadu District of Kerala. CCl_4 treated Chang cell line (normal human liver cells) were used for evaluation of the hepatoprotective activity of *Diplazium esculentum*. The methanolic extract of the *D. esculentum* was tested for its inhibitory effects on CCl_4-induced hepatotoxicity. Percentage viability of the Chang cell line was calculated. Administration of *Diplazium* extracts (100, 500 and 1000 µg) for 24 hrs significantly reduced the impact of CCl_4 toxicity in tested cell lines. Percentage viability ranged from 82 to 84%, was visible when pretreated with the extracts. The anti-inflammatory effect of *Diplazium* is most likely mediated through its ability to inhibit cyclooxygenase-2 (COX-2), lipoxygenase (5-LOX). The plant extracts at various concentration 100, 500 and 1000 µg were evaluated for COX-2 and 5-LOX inhibitory activity and methanol extract exhibited the highest inhibition of COX-2 and 5-LOX at concentration of 1000 µg. The results showed that *Diplazium esculentum* had considerable anti-inflammatory and hepatoprotective activities.

1. INTRODUCTION

A large number of plants have been claimed to have hepatoprotective activity. Nearly 231 plants have been claimed to possess liver protecting activity and in India, more than 87 plants are used in 33 patented and proprietary multi ingredient plant formulations[1]. Development of hepato protective drugs based on plants has been given importance in the global market. A number of studies reported that plant-derived extracts or plant derivatives such as phenolic compounds and flavonoids showed anti-inflammatory activity by controlling the levels of various inflammatory cytokines or inflammatory[2]. Moreover, many crude extracts and chemical constituents of plants have pharmacologic effects and clinical benefits. However, the claims of benefits of many plants or plant-based medicines marketed to the general population are only supported by empirical or preliminary scientific data[3].

Plant cells fundamentally are chemical factories and many possess a rich supply of therapeutically useful constituents. On numerous occasions, the folklore records of many different cultures have provided drug leads in plants[4]. *Diplazium esculentum* (Dryopteridacea), commonly called "Churuli" is considered being of high economic and medicinal value amongst the natives of wayanadu and adjoining areas. The plant contains steroids, triterpenoids, phenols, flavones and flavonoids[5]. This plant is used in folklore remedies for the treatment of various ailments such for curing of haemoptysis and cough[6]. Marginal anticancer activity was reported in the crude ethanol extract of the plant[7] and vegetative shoots posses antioxidant activity[8]. Dried rhizomes used as insecticides and the leaves are used as vegetables. Keeping these points in mind the aim of the study was to evaluate hepatoprotective and anti-inflammatory effect of the leaves of *Diplazium esculentum* in order to scientifically validate its folklore use in Wayanadu District of Kerala, India.

Habit of *Diplazium esculentum* (Retz.) Sw.

2. MATERIALS AND METHODS

2.1 Plant source and extraction procedure

The whole plant *Diplazium esculentum* were collected from Western Ghats region of Wayanadu. Twenty grams of powdered whole plant was taken in a soxhlet apparatus and 250 mL of 90% methanol was added. It was refluxed for 72 hrs and filtered through muslin cloth while hot. The methanolic extract was dried under vacuum.

2.2 Analysis of Hepatoprotective Activity

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), sulphorhodamine-B (SRB), ethidium bromide, phosphate buffered saline (PBS), triton X-100, acridine orange, olive oil, haematoxylin and eosin were procured from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Chang cell lines were procured from the National Centre for Cell Sciences (Pune) and cultured in DMEM medium supplemented with 5% (v/v) fetal bovine serum in a humidified incubator containing 5% CO_2 and 95% air at 37°C. Only cells in the exponential growth phase were used for experiments.

2.2.1 Cytotoxity Based Viability Assay

The cytotoxic effect was measured using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as per the methodology of Masters[9].

In MTT assay, exponentially growing cells were seeded in 96-well plates (104 cells/well in 100 µl of medium) and kept overnight for twenty four hours at 37°C in a humidified atmosphere of 5% CO_2. The cells were then exposed to toxicant (medium containing 15 mM CCl_4) along with and without 100, 500 and 1000 µg of methanol extract from *D. esculentum*. At the end of the period, cytotoxicity was assessed by estimating the viability of chang liver cells by MTT reduction assay. After one hour of incubation, the solvent solution from each well was removed by aspiration and replaced with 50 µl of MTT. The plates were shaken and incubated for three hours at 37^{o}C in a humidified 5% CO_2 atmosphere. The supernatant was removed and 50 µl of propanol was added and the plates were shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 540nm.

2.3 *In Vitro* Anti Inflammatory Activity

2.3.1 Lymphocyte Culture Preparation

Human peripheral blood lymphocytes were cultured in RPMI 1640 (HIMEDIA) media, supplemented with Penicillin. The culture was filtered using 0.2µm pore sized cellulose acetate filter under completely aseptic conditions. The culture was incubated for seventy two hours and then activated by adding 1µl lipopolysaccharides (LPS)[10].

2.3.2 Assay of Cyclooxygenase Inhibition

A standard drug (Indomethacin) was added to lymphocyte culture at a concentration of 1000 µg/ml from a stock of 100 mg/ml. Methanol extract of *D. esculentum* was added at concentrations of 1, 5 and 10 µl from a stock of 100 mg/ml, followed by incubation for twenty four hours[11]. Isolation was done by spinning lymphocytes with standard or sample at 6000 rpm for ten minutes. Supernatant was discarded and 200 µl of cell lysis buffer (1MTris-HCl, 0.25M EDTA, 2M Nacl, 0.5% Triton) was added. Incubated for thirty minutes at 4^{o}C and cyclooxygenase assay was done in pellets suspended in a small amount of supernatant[12]. The assay mixture contained Tris-HCL buffer, glutathione, haemoglobin & 0.25ml of lipoxidase enzyme. The reaction was started by the addition of arachidonic acid and terminated after twenty minutes. Incubation was done at 37^{o}C by the addition of 0.2 ml of 10% TCA in 1N HCL. Two ml of TBA was added and contents were heated in a boiling water bath for twenty minutes, cooled and centrifuged at 1000 rpm for three minutes. The supernatant was measured at 632 nm for COX activity. Percentage inhibition was calculated by using the following formula:

$$\text{Percentage of Inhibition} = \frac{\text{Absorbance of control-Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.3.3 Assay of Lipoxygenase Inhibition

Concentration of stock, sample and preparation of pellet was the same as cyclooxygenase inhibition assay. Lipooxygenase inhibition assay was done in pellet suspended in a small amount of supernatant of cell lysis buffer. Seventy mg of linoleic acid and equal weight of Tween 20 was dissolved in 4 ml of oxygen free water and mixed back and forth with the a pipette avoiding air bubbles[13]. Sufficient amount of 0.5 N NaOH was added to yield a clear solution and then made up to 25 ml using distilled water. The assay mixture contains 2.75 ml tris buffer at pH 7.4, 0.2 ml of sodium linoleate and 0.25ml of lipoxidase enzyme. The increase in OD was measured at 234 nm. The percentage inhibition was calculated by using the same formula as cyclooxygenase inhibition assay.

2.4 Statistical analysis

Experiments were done in triplicate. Data are expressed as means ± SEM. Student't' test were used to analyse mean difference between cells treated with CCl_4 and methanol extract, $P<0.001$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Hepatoprotective activity

The longevity of an organism clearly depends on its individual parts and their effective organization. The tetrazolium salt (3-(4, 5 dimethylthiazole –2 yl)-2, 5 diphenyl tetrazolium bromide) is taken up into the cells and reduced in a mitochondria dependent reaction to yield a blue coloured formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. The product is librated and can be readily detected by solubilisation of cells and quantified by simple colorimetric method. An indication of mitochondrial integrity is determined by the ability of cells to reduce MTT and activity which in turn may be interpreted as a measure of viability and/or cell number. The assay has therefore been adopted for use with cultures of exponentially growing cells[14]. Determination of their ability to reduce MTT to the formazan derivative after exposure to test compounds compared to the control situation, enables the relative protection of test chemicals to be assessed. From the above results it is confirmed that cells which are exposed only with toxicant CCl_4 showed a percentage viability of 42% while cells which are pretreated with extract showed an increase in percentage viability and the results were highly significant ($P<0.001$, when compared to CCl_4 intoxicated cells). The percentage viability ranged from 82 to 84% cell viability, when pretreated with the extracts.

Table 1: Protective effect of given extracts on CCl_4 induced toxicity in Chang liver cells.

Treatment	OD at 540nm	% viability*
Control(untreated cells)	1.701	100
CCl_4	0.324	$19.05 \pm 0.27^{+}$
Silymarin (Standard)		
100µg	0.60	$77.9 \pm 0.94^{++}$
500µg	0.65	$84.41 \pm 0.31^{++}$
Sample concentration		
100 µg	0.6845	$40.24 \pm 0.47^{++}$
500 µg	1.232	$72.42 \pm 1.12^{++}$
1000µg	1.616	$95 \pm 0.08^{++}$

*Average of three independent determinations, values are mean ± S.E.M. + = $P < 0.001$, when compared to untreated cells. ++ = $P < 0.001$, when compared to CCl_4 intoxicated cells.

Figure (1-5): Effect of methanol extract of *Diplazium esculentum* in CCl_4 treated Chang liver cells

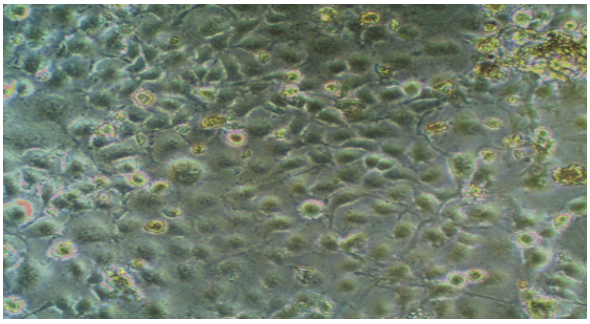


Fig 1: Control (without treatment)

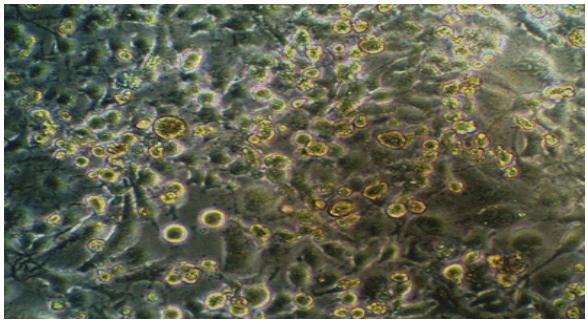


Fig 2: Treatment of Cell lines with CCl_4

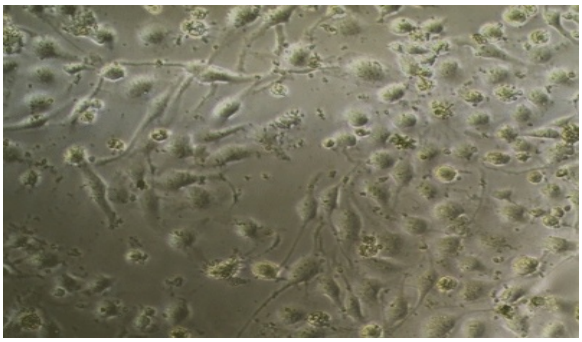


Fig 3: CCl_4 + 100µg ME of *D. esculentum*

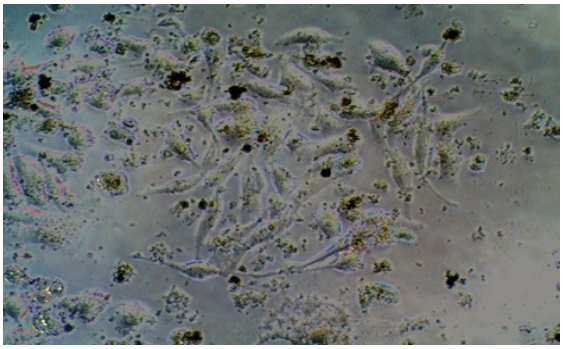


Fig 4: CCl_4 + 500µg ME of *D. esculentum*

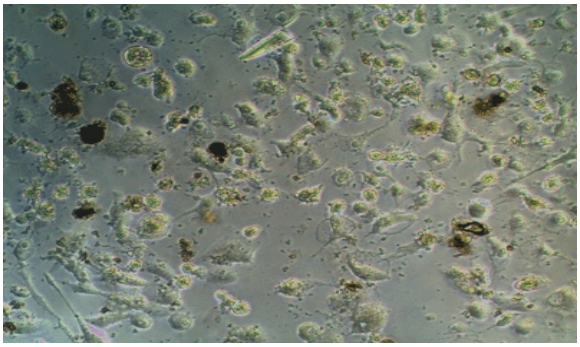


Fig 5: CCl_4 + 1000µg ME of *D. esculentum*

3.2 Anti-inflammatory activity

The anti-inflammatory properties of bioactive molecules have been attributed, at least in part, to suppression of prostaglandins (PGs) synthesis. For over three decades the involvement of PGs and other eicosanoids in the development of human cancer has been known. Importantly, in human beings and experimental animals an increase in PG synthesis might influence tumor growth. Numerous studies have illustrated the effect of PG synthesis on carcinogen metabolism, tumor cell proliferation, and metastatic potential[15]. Conversion of Arachidonic acid to PGs is catalyzed by key enzyme Cyclooxygenase (COX), which exists in two different isoforms, designated COX-1 and COX-2. COX-1 is a constitutive isoform present in most tissues and its

inhibition results in serious effects such as peptic ulceration or impairment of renal blood flow. In contrast, COX-2 is constitutively expressed only in brain and spinal cord tissue. COX-2 overexpression has been implicated in the carcinogenesis of tumors of the colon, rectum, breast, head and neck, lung, pancreas, stomach, and prostate[16]. There is growing evidence that inhibitors of COX-2 activity are useful for treating inflammation and preventing or treating cancer[17]. LOX metabolites have also been shown to stimulate the spreading of tumor cells, promote tumor cell adhesion and augment metastatic potential[18].

In present study, COX-2 and 5- LOX pathway was stimulated by using mouse peritoneal macrophages. Methanol extract of *Diplazium esculentum* is capable of exerting inhibitory action on enzymes of the arachidonate cascade, although they do not interfere in the COX-1 pathway, they inhibit COX-2 by 61.76 % inhibition. Methanol extracts of *D. esculentum* exert a preferential effect on the 5-LOX pathway, with an inhibition rate of 44.44%.

Fig 6: Cyclooxygenase Inhibition in methanol extract of *Diplazium esculentum*

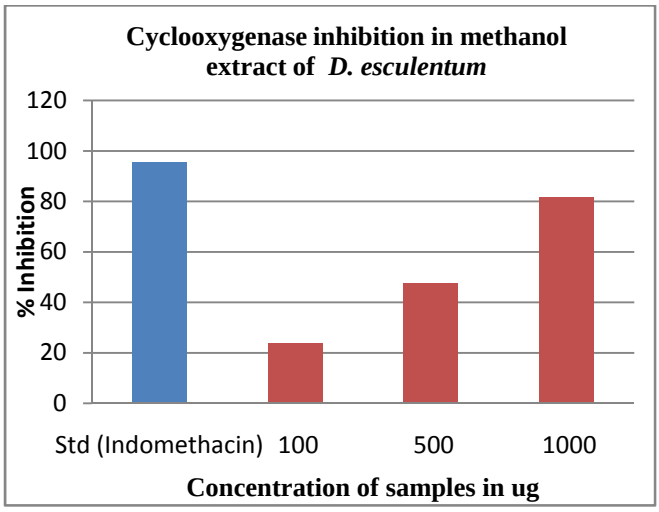
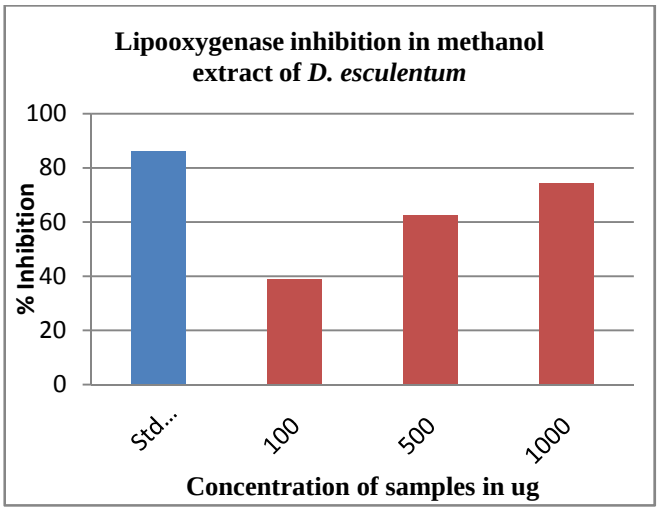


Fig 7: Lipooxygenase Inhibition in methanol extract of *Diplazium esculentum*



4. CONCLUSION

The present study concludes that the plant *Diplazium esculentum* has appreciable anti-inflammatory potential, which supports the claim of local people, and more work in this direction has to be done to confirm its utility in higher models. The above study indicates positive hepatoprotective activity of the extract *D. esculentum in vitro*, against CCl_4 induced hepato toxicity and anti-inflammatory activity.

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