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RESEARCH ARTICLE

Anti-Inflammatory and Anticancer Activity of *Pteris cretica* Whole Plant Extracts

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ABSTRACT

The present study focuses on the use of n-hexane, chloroform, ethyl acetate and aqueous extracts of *Pteris cretica* to confirm its *in-vivo* anti-inflammatory properties and *in-vitro* cytotoxic profile. The anti-inflammatory study was conducted by carrageenan-induced edema test at different doses of *Pteris cretica* extracts (250 and 500mg/kg body weight). At 250 mg/kg dose of chloroform, n-hexane, aqueous and ethyl acetate extracts expressed maximum anti-inflammatory activity at the 2nd hour with 29.65, 29.06, 27.55 and 26.04% respectively by inhibiting carrageenan-induced edema. Moreover, 500 mg/kg concentration of chloroform extract of *Pteris cretica* expressed efficient anti-inflammatory activity with 45.3% of inhibition in edema; whereas n-hexane, aqueous and ethyl acetate extracts inhibit the edema at 35.34, 29.06 and 27.55% respectively. MTT assay was used to determine the cytotoxic potential of *Pteris cretica* extracts on HeLa, and BHK-21 cell lines. Chloroform and aqueous extracts expressed the cytotoxic activity with IC_{50} of 31.48 and 34.26 µg/mL respectively against HeLa cell lines. However, both chloroform and aqueous extracts exhibit IC_{50} 108.50 and 55.76 µg/mL against BHK-21 cell line respectively. Chloroform extract is less cytotoxic against BHK-21 cell line as compared to aqueous extract. Ethyl acetate extract was found to be toxic for both cell lines. Based upon the above results, isolation, identification and purification of phytochemicals from *Pteris cretica* extracts is highly recommended as a possible extension of the current work.

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INTRODUCTION

Traditionally hundreds of herbs are used in folk medicine to treat various diseases (Hossen *et al*., 2016; Liaqat *et al*., 2016; Abbas *et al*., 2017a; Hassan *et al*., 2017; Tahir *et al*., 2017). Presently alternative medicines become more integral portion in the modern world due to its novelty and structural diversity (Abbas *et al*., 2017b). According to WHO (World Health Organization, 2002); approximately 80% of the population are using herbal medicine in developing countries because of their cost-effectiveness and less adverse effects' (El-Wahab *et al*., 2013).

Pteris cretica is a perennial herb, belong to family *Pteridacene*. The common name of *Pteris cretica* is Pata. Its major abundance in Pakistan found in the Pir Nasoora park, Azad Jammu and Kashmir (Saima *et al*., 2010).

Pteris cretica belongs to lower vascular plants group called *Pteridophyta* having true leaves, vascular bundles, without flowers and seeds (Shil and Choudhury, 2009). In 1935, Caius was the first person who studied *Pteridophyta* in India and reported its medicinal uses as emollient, anthelmintic in cough and in wound healing etc (Mannan *et al*., 2008).

The uncontrolled or abnormal growth of cells is known as cancer. Some medicinal plants and their phytoconstituents are being increasingly recognized as useful complementary treatments for cancer. Many useful compounds like Taxol, Docetaxel, Deacetylbaccatin III, Paclitaxal, Podophyllotoxin, Demethylpodophylotoxin, α-peltatin, β-Peltatin, Topotecan, Vinblastine, Vindesine, Vinoselbine and Vincristine have been isolated from the

plant source and they have played a vital role in anticancer therapy (Kaur *et al*., 2011).

The intricacy with respect to inflammation reaction to different medical issues in the greater part of the populace having an adverse impact on physiological and pathological condition. Inflammation is a sever response by living tissue to any kind of injury which protects the host from pathogens. The primary indicators of inflammation are pain, redness, heat and swelling (Sosa *et al*., 2002). In inflammatory conditions, a huge number of demagogic mediators' leucocytes accumulate at the site of inflammation. Acute inflammation is initial response of the body to harmful stimuli. The important inflammatory mediators such as Cyclooxygenase-II (COX-II), prostaglandins and nitrogen oxide play a key role in the different steps of the signaling pathway of inflammation. The inhibition of these inflammatory mediators is the primary target of anti-inflammatory drugs to cure disease related to inflammation (Michael *et al*., 2003; Mehmood *et al*, 2016). Currently non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are used to cure inflammation, but the chronic use of these agents carry the high risk of stomach ulceration and gastrointestinal bleeding (Pilotto *et al*., 2003).

In-vitro toxicity studies of drugs have increased critical significance and use as a parameter for the assessment of safety profile of various medications. The carcinogenic prospective along with the mutagenic effect has been observed in different natural compounds. MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is the ideal method for identification of cytotoxic abilities of plant extracts and drugs. MTT assay concludes the cells viability upon exposure of cytotoxic drugs, via measuring the formazan dyes color which is made by an enzyme exhibits in mitochondria (Khasawneh *et al*., 2011; Stockert *et al*., 2012; Sharif *et al*., 2016).

This study comprises extracts (n-hexane, chloroform, aqueous and ethyl acetates) of *Pteris cretica* for assessment of anti-proliferating and anti-inflammatory effect of all extract.

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals and reagents used in the study were of analytical grade.

Collection and authenticity of plant: The whole plant was collected from the wild forest of Swat, Pakistan. The plant was spotted by Voucher No: GC Herb Bot 2984 in the herbarium of Government College University, Lahore, Punjab, Pakistan.

Preparation of sample & extraction: The whole plant was washed with tap water and shade dried for 10 days in order to avoid enzymatic degradation. The fully dried plant was ground in the electric herbal grinder and stored in clean airtight, glass container. The powder plant (500 g) was extracted with n-hexane, chloroform, ethyl acetate and distilled water to prepare extracts by using Soxhlet apparatus (Ahmad *et al*., 2009). The sequential extraction process was last till solvent appeared colorless in the thimble (7-14 days). All extracts were filtered and dried on the rotary evaporator at 40°C under abridged pressure to get resinous extracts of n-hexane, chloroform, ethyl acetate and water. Dried extracts were weighed and stored in glass vials in the refrigerator.

Animals: 14 Days prior to the experiment, 50 Wistar rats (150-200g) of the same batch were obtained from Post Graduate Medical Institute, Lahore, Pakistan and kept under controlled temperature (25±1°C). They were fed on the balanced diet and free access to water in their cages according to twelve hourly day and night interval.

Animal groups: Rats were randomly divided into 10 groups; (n=5). Each group named as following:
Group I: (Negative control) received 1ml of Normal saline, orally. Group II: (Positive control) received Diclofenac sodium (50 mg/kg). Group III, IV, V and VI received 250 mg/kg whereas Group VII, VIII, IX and X received 500 mg/kg of n-hexane, chloroform, aqueous and ethyl acetate extracts respectively.

Inflammation measurement: Two concentrations (250 and 500 mg/kg body weight) of extracts were given orally to the respective groups in solution form in normal saline, one hour before carrageenan injection (0.1 ml of 1% w/v suspension). One hour after the administration of oral dose, the carrageenan was injected in the right-paw of the rats. The inflammation was measured in terms of ml by displacement of normal saline due to edema with the help of digital plethysmometer immediately before and after (0, +1, +2, +3, +4 h) carrageenan injection (Akhtar *et al*., 2016). The percentage inhibition of edema was calculated with following formula;

$$\text{Percentage inhibition of paw edema } = \left(\frac{\text{Vc} - \text{Vt}}{\text{Vc}}\right) \times 100$$

Vc represents the average increase in paw volume of the control group at a given time; Vt was the average volume of the paw of treated groups at the same time (Akhtar *et al*., 2016).

MTT analysis: MTT assay was performed according to (Sharif *et al*., 2017) with slight modifications. MTT assay was used to measure the cell proliferation rate. Two different cell lines HeLa (cancerous) and BHK-21 (normal) were used. The HeLa cells were incubated for 24h at 37°C, on the other hand, BHK-21 cells were incubated for 48h and exposure with extracts (200µl) was given for 24h and 48h respectively. The cells monolayer was washed with PBS (pH 7.4), 100µl medium containing 25µl MTT solution was tipped in each well of 96 well plate and incubated for 3 hours. MTT solution was converted into purple colored formazan in living cells, was solubilized with 10% sodium dodecyl sulphate (SDS). Finally, the absorbance of the solution was observed at 570 nm with Enzyme-Linked Immunosorbent Assay ELISA reader. Following equation was used to calculate the cell survival percentage (CSP) (Sharif *et al*., 2017).

Cell survival percentage (CSP) = (Mean optical density of test chemical – Mean optical density of negative control) × 100) / Mean optical density of positive control.

Statistical analysis: Statistical analysis of data was concluded by two-way ANOVA. Cell survival percentage was evaluated by non-linear regression analysis on GraphPad prism 7.0.

RESULTS

Anti-inflammatory: Ethyl acetate extract, chloroform, n-hexane and aqueous extract of *Pteris cretica* (250 mg/kg) expressed significant anti-inflammatory properties (P<0.0001) till the 3rd hour of administration as compared to group II, positive control group, Fig. 1a. However, n-hexane, ethyl acetate and aqueous extracts did not show anti-inflammatory properties against group II (positive control group) at 4th h of administration, whereas only group VI (250 mg/kg chloroform extract of *Pteris cretica*) have significant difference of anti-inflammatory properties with group II (positive control group) at 4th hour of administration. All the four extracts of *Pteris cretica* possessed significant anti-inflammatory activity as compared to the negative control group till the 4th h of administration Fig. 1a. Mean paw size expressed as mean±SD and percentage inhibition of paw edema are depicted in Table 1.

Moreover, ethyl acetate, n-hexane and aqueous extract of *Pteris cretica* (500mg/kg) expressed anti-inflammatory properties at the significant level (P<0.0001) till the 3rd h of extract administration as compared to group II (positive control group). Although chloroform (500 mg/kg) extract group did not show the significant difference in anti-inflammatory activity with group II (positive control group) during whole experiment time as illustrated in Fig. 1b. All extracts of *Pteris cretica* (500 mg/kg) exhibited significant difference with negative control till 4th h of administration Fig. 1b.

The results of present study exhibit that *Pteris cretica* extracts possesses promising anti-inflammatory properties. Furthermore, chloroform extract has highest anti-inflammatory activity in both doses. The maximum decrease in paw edema by chloroform extract at 250 and 500 mg/kg was 29.65 and 45.23% respectively, were comparable with diclofenac sodium 49.30% as mentioned in Table 1.

Cytotoxicity: Cell survival percentage (CSP) of the four extracts of *Pteris cretica* in HeLa (cancerous) and BHK-21 (non-cancerous) cells lines followed a dose-dependent fashion as depicted in Table 2a & b. IC_{50} is defined as the concentration at which 50% of cells were inhibited, it was calculated by using GraphPad prism 7.0. According to results chloroform extract exhibited IC_{50} at 31.48 and 108.50 µg/ml (Fig. 2a & b) for HeLa and BHK-21 respectively. Whereas IC_{50} of aqueous extract was 34.26 and 55.76 µg/ml in HeLa and BHK-21 respectively (Fig. 2a & b). The chloroform extract possessed higher anti-proliferative potential against the cancerous cell line (HeLa) at the lower concentration with better safety profile against non-cancerous cell line (BHK-21).

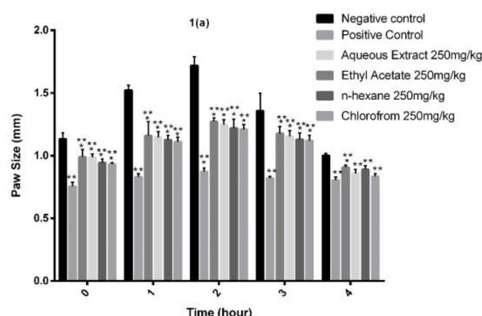
DISCUSSION

The anti-inflammatory activity of *Pteris cretica* was studied by using carrageenan prompted rat paw edema testing at different doses of n-hexane, chloroform, aqueous and ethyl acetate extracts of *Pteris cretica* (250mg/kg and 500mg/kg). Inflammation has two-phase events in which the first phase is recognized by the release of histamine and serotonin. The second phase of edema is due to the release of protease, prostaglandins and lysozymes. Most of the anti-inflammatory drugs are efficient in the second phase of inflammation (Ahmad *et al*., 1992; Gupta *et al*., 2005).

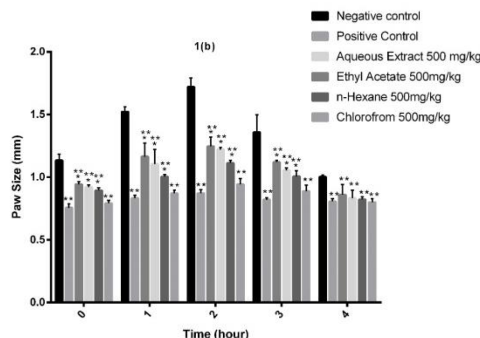
Carrageenan-induced edema method are most extensively used method for anti-inflammatory studies of drugs. It involves the synthesis or release of inflammatory mediators such as; prostaglandins, bradykinins, histamine and serotonin at the injured site. Development of edema particularly due to induction of carrageenan is normally associated with initial exudative phases of inflammation (Asongalem *et al*., 2004; Hossain *et al*., 2017). Carrageenan edema is a multiple intervened singularity that releases different type of mediators. The carrageenan-induced paw edema model is known to be sensitive to the effect of NSAIDs which primarily inhibits cyclooxygenase involved in the synthesis of prostaglandins (Hemamalini *et al*., 2010; Akhtar *et al*., 2016). Furthermore, carrageenan-induced edema method is a substantial prognostic test for anti-inflammatory studies, the results of this study suggested that *Pteris cretica* can be effective in the treatment of inflammation (Hossain *et al*., 2017).

Table 1: Data represent Mean ± SD of rat paw size after administration of *Pteris cretica* extracts and percentage of paw edema inhibition with reference of time (n=5)

Treatment	Dose (mg/kg)	Paw size (mm) after administration of plant extract + S.D (Percentage of paw edema inhibition)				
		0 h	1	2 h	3 h	4 h
Normal Saline	10 ml	1.134 ± 0.048	1.522 ± 0.04	1.72 ± 0.07	1.358±0.141	1.002 ± 0.16
Diclofenac sodium	50 mg	0.756±0.032 (33.33%)	0.832±0.25 (45.33%)	0.872±0.28 (49.30%)	0.82±0.16 (39.61%)	0.806±0.23 (19.56%)
n-Hexane extract	250 mg	0.944±0.030 (16.75%)	1.126±0.036 (26.01%)	1.220±0.072 (29.06%)	1.130±0.052 (16.78%)	0.890±0.032 (11.17%)
	500 mg	0.894±0.021 (21.16%)	1.004±0.018 (34.03%)	1.112±0.022 (35.34%)	1.006±0.045 (25.92%)	0.822±0.022 (17.96%)
Chloroform extract	250 mg	0.932±0.013 (17.81%)	1.110±0.037 (27.06%)	1.210±0.038 (29.65%)	1.120±0.042 (17.52%)	0.834±0.023 (16.76%)
	500 mg	0.792±0.24 (30.15%)	0.870±0.027 (42.38%)	0.942±0.047 (45.23%)	0.888±0.049 (34.60%)	0.800±0.064 (20.15%)
Ethyl acetate extract	250 mg	0.990±0.058 (12.69%)	1.160±0.111 (23.78%)	1.272±0.029 (26.04%)	1.176±0.056 (13.37%)	0.908±0.015 (9.38%)
	500 mg	0.942±0.022 (16.93%)	1.164±0.108 (23.52%)	1.246±0.074 (27.55%)	1.120±0.014 (17.52%)	0.860±0.080 (14.17%)
Aqueous extract	250 mg	0.986±0.027 (13.05%)	1.146±0.046 (24.70%)	1.246±0.040 (27.55%)	1.154±0.046 (15.02%)	0.860±0.032 (14.17%)
	500 mg	0.920±0.019 (18.87%)	1.104±0.117 (27.46%)	1.220±0.019 (29.06%)	1.054±0.019 (22.38%)	0.832±0.064 (16.96%)



The data was expressed as Mean±S.D and analysed by Two way ANOVA (Tukeys multiple comparison test).
* p<0.0001 statistically significnat when all the treatment groups were compared with positive control group at 0 hour, 1 hour, 2 hour, 3 hour and 4 hour.
** p<0.0001 statistically significnat when all the treatment groups and positive control were compared with negative control control group at 0 hour, 1 hour, 2 hour, 3 hour and 4 hour.



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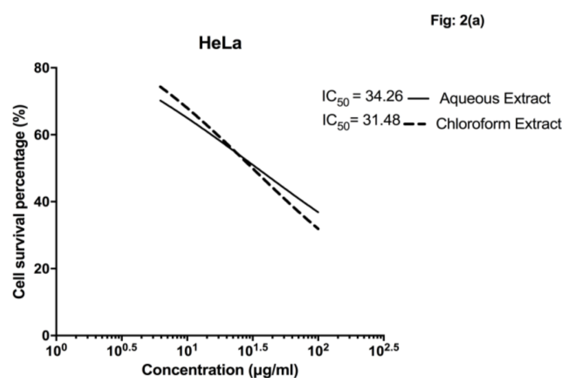
** p<0.0001 statistically significnat when all the treatment groups and positive control were compared with negative control control group at 0 hour, 1 hour, 2 hour, 3 hour and 4 hour.

Fig. 1: Illustrates the mean paw size of rat after administration of control and *Pteris cretica* extracts, 1(a) graphical representation of *Pteris cretica* extracts at dose of 250mg/kg of body weight, 1(b) graphical representation of *Pteris cretica* extracts at dose of 500 mg/kg of body weight.



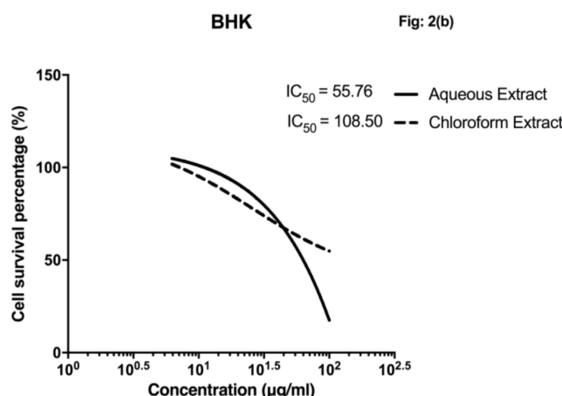


Fig. 1: MTT assay of *Pteris cretica* aqueous and chloroform extracts at different concentrations; 2(a) illustrates the inhibitory concentration (IC50) of aqueous and chloroform extract in HeLa cell lines, 2(b) illustrates the inhibitory concentration (IC50) of aqueous and chloroform extract in BHK-21 cell lines.

Table 2a: Data represents the cell survival percentage of BHK-21 cell line at the different concentrations of n-hexane, chloroform, ethyl acetate and aqueous extracts of *Pteris cretica*

	BHK-21 Cell survival percentage				
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml
n-Hexane Extract	54.01	74.04	77.99	84.18	91.81
Chloroform Extract	57.59	60.51	79.89	95.07	99.62
Ethyl acetate Extract	11.17	25.08	23.05	98.92	92.10
Aqueous Extract	18.97	53.93	98.69	98.65	99.36

Table 2b: Data represents the cell survival percentage of HeLa cell line at the different concentrations of n-hexane, chloroform, ethyl acetate and aqueous extracts of *Pteris cretica*

	HeLa cell survival percentage				
	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml
n-Hexane Extract	45.25	47.03	52.99	58.24	70.46
Chloroform Extract	31.99	41.62	55.82	62.82	75.01
Ethyl acetate Extract	34.56	40.74	43.72	51.02	77.08
Aqueous Extract	39.7	46.18	49.05	58.70	76.01

Carrageenan-induced edema method in most extensively used method for anti-inflammatory studies of drugs. It involves the synthesis or release of inflammatory mediators such as; prostaglandins, bradykinins, histamine and serotonin at the injured site. Development of edema particularly due to induction of carrageenan is normally associated with initial exudative phases of inflammation (Asongalem *et al.*, 2004; Hossain *et al.*, 2017). Carrageenan edema is a multiple intervened singularity that releases the different type of mediators. The carrageenan-induced paw edema model is known to be sensitive to the effect of NSAIDs which primarily inhibits cyclooxygenase involved in the synthesis of prostaglandins (Hemamalini *et al.*, 2010; Akhtar *et al.*, 2016). Furthermore, carrageenan-induced edema method is a substantial prognostic test for anti-inflammatory studies, the results of this study suggested that *Pteris cretica* can be effective in the treatment of inflammation (Hossain *et al.*, 2017).

In plants, flavonoids are performing a multiplicity functions like protection against ultra-violet, re-absorption of mineral as well as pollination and tolerance to A-biotic stresses etc. Flavonoids represent a significant group of plant secondary metabolites with unpredictable phenolic structures which are found in bark, fruits, flower, grains, roots, tea, vegetables and wine (Batra & Sharma, 2013). Studies suggested that different secondary metabolites like Flavonoids, Tannins, Saponins and Steroids are considered, responsible for antioxidant, anticancer, hypolipidemic, antihypertensive and anti-inflammatory activities. Previously studies of *Pteris cretica* confirmed the presence of flavonoids content (Gracelin *et al.*, 2013; Saleem *et al.*, 2016; Hossain *et al.*, 2017). Flavonoids are naturally occurring compound and have anti-inflammatory action during different phases of inflammation such as exudative and proliferative phase of inflammation (Rathee *et al.*, 2009; Hossain *et al.*, 2017).

Flavonoids are responsible for the anti-inflammatory activity of *Pteris cretica* and flavonoids presence in *Pteris cretica* is confirmed from our previous studies (Saleem *et al.*, 2016).

Diclofenac sodium used in this study as a positive control. It is demonstrated as NSAIDs (non-steroidal anti-inflammatory drugs) and acts by its activity through the inhibition of prostaglandin synthesis by limiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-

2) with comparative equipotency (Gan, 2010). The data represented that the ethyl acetate extract at the dose of 500 mg/kg showed a significant reduction ($P<0.0001$) in edema and faster rate of inhibition as compared to 250 mg/kg dose. Perhaps it can be logically concluded that the inflammation inhibitory effect of chloroform extract, aqueous extract, ethyl acetate extract and n-hexane extract of *Pteris cretica* may be due to inhibition of cyclooxygenase enzyme in same manner as diclofenac sodium does.

Presently, the unpremeditated connection among inflammation and cancer is broadly acknowledged. It is now manifested that inflammatory mediators/ inflammatory cells play an influential tumor promoter in early stages of the neoplastic process and provide a potential atmosphere for cancer growth. Chronic inflammation has strongest link between multiple malignancies such as; inflammatory bowel disease progress to colorectal carcinogenesis like chronic ulcerative colitis and Crohn's disease, liver carcinoma originates from hepatitis C infection. It is believed that chronic inflammation promotes the infectious state of disease into cancer via DNA damages. The overexpression of macrophage migration inhibitory factor (MIF) by inflammatory cells induce the aggravating of DNA damages. Furthermore, in infiltrated tissues, p53 regulatory function is chronically bypassed and provided an ample environment for oncogenic mutations in DNA. So, possession of anticancer activity along with the anti-inflammatory activity in *Pteris cretica* extracts is beneficial for cancerous patient (Coussens and Werb, 2002).

Although cytotoxicity of *Pteris cretica* was not reported previously but its genus constituents (*2R, 3R*-pterosin L O-β-D glucopyranosade and pterosin-B) have been documented as anti-proliferative agents (Chen *et al.*, 2008).

The HeLa (cancerous) and BHK-21 (non-cancerous) cell lines were exposed to various concentrations of n-hexane, chloroform, ethyl acetate, aqueous extracts of *Pteris cretica*. Chloroform extract is most effective in inhibiting HeLa cell lines followed by ethyl acetate, aqueous and n-hexane extracts. Ethyl acetate extract is toxic for both cell lines. All *Pteris cretica* extracts showed dose-dependent cytotoxic effects, which proposed the therapeutic potential of this plant. These results are in accordance with the previous investigations, where *Pteris multifida* ethanolic and methanolic leaf extracts depict cytotoxicity against HeLa and NCl-H460 cell lines (Son and Thao, 2014).

Conclusions: MTT assay unfolded potential cytotoxic and anti-proliferative activity of *Pteris cretica* extracts. Initial screening showing cytotoxicity of the transformed cell line may depict possible anti-proliferative and anti-cancer effect of *Pteris cretica*. The confirmation of anti-proliferative and cytotoxicity properties of *Pteris cretica* extracts open a new horizon for further testing to treat cancer with potential anti-inflammatory properties.

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Authors contribution: FS-project designing; KK-student, SA-provided the cell line and helped in anticancer activity; SA-statistical analysis of antioxidant data; SA-guided in extraction and phytochemical analysis; MAAM-statistical analysis of anti-inflammatory data; BA–guided in anti-inflammatory activity; MR-guided in DPPH method; SP-preparation of manuscript; AS–statistical analysis of MTT data, preparation of manuscript and proof reading of paper.

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