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In vitro cytotoxicity of the polar extracts of *Potentilla fulgens* L. against human cancer cell lines: Detection and isolation of bioactive phenolics

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ABSTRACT

Potentilla fulgens L., a locally used medicinal plant with reported hypoglycemic, antihyperglycemic, and anti-tumor properties was studied. The polar extracts of P. fulgens L. were assessed and found to induce cytotoxic response at different concentrations against a panel of human carcinoma cells such as THP-1 leukemia, HEP-2 liver, OVCAR-5 ovary, A-549 lung, PC-3 prostrate, and SF-295 neuroblastoma. Ability of the polar extracts to induce cytotoxicity was compared with two known anticancer drugs, 5-flurouracil and mitomycin. We report, the polar fractions of P. fulgens L. show in vitro cytotoxic activity against various human cancer cell lines. The anti-oxidant property, assessed by DPPH and H_2O_2 scavenging methods, showed significant activities in the polar extracts. Isolation and characterization of the phenolics present in the polar fractions, using chromatographic and spectroscopic techniques, led to the detection of some polyphenols such as (+)-catechin, ellagic acid, kaempferol and quercetin.

Keywords: Traditional medicinal herb; *Potentilla fulgens* L.; bio-assay guided isolation; cancer cell lines: cytotoxicity.

INTRODUCTION

Potentilla Lenn. Genus, a member of the family Rosaceae and subfamily Rosoideae, comprises approximately 500 species which include perennial, rarely biennial, and annual herbs besides small shrubs with rhizomes. In their natural habitat, they are mainly distributed in temperate, arctic and alpine zones of the northern hemisphere. A few species of this family are also found in high mountain regions of the tropics, and in South America. Many plants of the genus *Potentilla* have been recognized to have wide ranging medicinal properties including antioxidant, anthelmintic, digestive stimulant, anti-inflammatory, antimicrobial, hypolipidemic, hypoglycemic, anti-mutagenic, antidiarrhoic, anti-ulcerogenic, anti-neoplastic, antiviral, antimicrobial, antihyperglycemic, anti-inflammatory, spasmolytic, hepatoprotective, and anti-carcinogenic [1,2]. *Potentilla fulgens* L. finds habitat in the western Himalayan region, and in higher altitudes (1500–2000 MSL) of Khasi and Jaintia Hills, Meghalaya, India. Traditionally pieces of roots are chewed along with betel, composed of raw areca nut (*Areca catechu*), locally called ‘‘Kwai’’, and betel leaf (*Pipper betel*) and is reported to exhibit antihypoglycemic, antihyperglycemic, antioxidant and anti-helmenthic properties [3,4,5].

Phenolic constituents are commonly found in both edible and inedible plants, and they have been reported to have multiple biological effects [6]. Medicinal plants containing phenolic compounds are used in the domains of medicine, nutrition, flavouring agent, beverages, dye, repellents, fragrances, and cosmetics [7]. They are finding increasing interest in the food industry because they retard oxidative degradation of lipids by destroying the reactive

oxygen species (ROS), and thereby improve the quality and nutritional value of food. The ascending trend on the use of plant derived natural antioxidants may also be attributed to the health and safety concern associated with the use of synthetic oxidants in food products [8]. Therefore, studies of natural antioxidants in foods or medicine are getting huge importance in recent years as an alternative to synthetic antioxidants.

It is worthwhile to mention that reactive oxygen species (ROS), including radicals and oxidants play various roles in the development of cancer. ROS can directly damage DNA, activate transcription factors, or active kinases and each of these changes may activate various genes including oncogenes. Gene activation may also be mediated by cytokines released in response to ROS or from phagocytic cells [9]. The fact that several nutritive dietary anticarcinogens are well-known antioxidants and/or radical scavengers, had led to growing interest in natural phenolic constituents present in medicinal and dietary plants that help attenuate oxidative damage [10, 11]. The importance of the effects of nutrition and dietary intake on cancer is well established [12, 13]. In continuation of our research [3, 5], we undertook a plan to examine the *in-vitro* cytotoxicity of various polar extract of *P. fulgens* against various human cancer cell lines. Since the ROS play an important role in the development of cancer (vide infra), we investigated antioxidant potential of some polar extracts of the said plant that showed significant antitumor activity.

In the present study it was intended to isolate and characterize the phenolic constituents which will help to understand the pharmacological properties that is our associated with the polar extracts of *P. fulgens*, by high performance liquid chromatography associated with diode array detection (HPLC-DAD), infra-red and mass spectrometry and nuclear magnetic resonance analysis (NMR) techniques.

EXPERIMENTAL SECTION

General experimental procedures

IR spectra were recorded on a Perkin Elmer spectrum BX spectrometer with a KBr pellet. Nuclear magnetic resonance (NMR) spectra were recorded on a Brucker DPX-200 and Bruker Avance II 400 spectrometer operating at 200 MHz (^{1}H), 400MHz(^{1}H) and 100MHz(^{13}C). The chemical shifts were expressed in ppm and tetramethylsilane (TMS) was used as an internal standard. Mass spectra were recorded on an Agilent Technologies 6538 UHD Accurate-Mass Q-TOF LC/MS spectrometer (Agilent Technologies, Santa Clara, CA), Waters ZQ-4000 LC-MS, and Tecan Sunrise™ Microplate Reader, and HetoLyolab 3000 Freeze dyer was also used for fluorescence assay and to dry-freeze the plant sample. Sephadex LH-20 (Pharmacia, Uppsala, Sweden), Silica gel (100–200 and 200–300 mesh 43–60µm; Merck, Darmstadt, Germany), were used for gel and column chromatography. Thin layer chromatography (TLC) analyses were performed with silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany). Human ovarian cancer cell line (OVCAR-5), human leukemia cell line (THP-1), and human neuroblastoma cancer cell line (SF-295) were procured from National Centre for Cell Sciences (NCCS), Pune, India. Human lung carcinoma cell line (A-549) and human liver cancer cell line (Hep-2) were obtained from National Cancer Institute, Frederick, U.S.A. Human prostate cancer cell line (PC-3) were obtained from National Cancer Institute (NCI), Bethesda, USA.

Plant material

Potentilla fulgens was collected from Shillong peak area of Meghalaya, India and identified with the voucher specimen (voucher number 464) by Curator Dr. P B Gurung, in the herbarium of Department of Botany, North Eastern Hill University, Shillong. The roots were separated from the main plant, weighed, washed, shredded and dried under shade. Dried roots were grounded to get a homogenous powder and stored at -80 ^{0}C before preparation of the extracts for analysis.

Preparation, fractionation and isolation of the polar extracts. The dried root powder (5.5 kg) of *P. fulgens* was macerated with 90% methanol (MeOH,v/v) (1.0L x 5) for six days, filtered, evaporated in rotavapor under vacuum and lypholizied to obtain the MeOH extract (859.0g). The methanol extract obtained was suspended in distilled water and successively partitioned with *n*-butanol, to obtained the butanol extract. The dichloromethane: methanol (DCM-MeOH 1:1,v/v) extract (286.0 g) was prepared separately from the root powder (0.8 kg) after defatting with 100ml petroleum ether (60-80 ^{0}C), for three times and extracted with DCM-MeOH (v/v) for 48 h by maceration method. The resulting mixture was filtered and evaporated in rotavapor under vacuum. The extract(s)/fractionate obtained were then stored at -80 ^{0}C and used for subsequent studies.

The *n*-butanol (90.0 g) soluble fraction was fractionated through repeated column chromatography using silica gel (60-120 & 200-300 mesh) with a gradient solution of dichloromethane-methanol (DCM-MeOH) as eluent with increasing proportions of methanol into different fractions. The fractions were further purified by sephadex LH-20 using methanol as eluent while monitoring by thin layer chromatography (TLC) using silica gel 60 F_{254} plates. The isolation of the *n*-butanol fraction yielded known compounds, as (+) -catechin (15.7 mg, 1), quercetin (14.0 mg, 2),

ellagic (4.0 mg, 3) and kaempferol (11.4 mg, 4), but not previously reported in *P. fulgens.*

Identification and quantification of phenolic compounds by HPLC analysis. HPLC-DAD analysis was performed with a Waters HPLC system equipped with 600 quaternary gradient pump, auto sampler, PDA detector, and Empowers software. The chromatographic separation was performed on a LiChroCART®125–3 Purospher® RP-18 (5 µm) Merck Labs column thermostatic at 30 °C. Elution of standards and extracts were performed using gradient solvent programming at a flow rate of 1.0 mL/min for 75 min. The mobile phase consisted of acetonitrile (A) and 5% acetic acid (v/v) in water (B) and was run in gradient mode. Initially, 92% of A and 8% of B was run for 5 min, then B was raised to 75% and A was change to 25% from 65 to 67 min, and A was again raised to 92% from 65 to 75 min. The column was then re-equilibrated with the initial conditions for 5 min before the next injection. The detection wavelength was set at λ 280 nm for the first run and at λ 340 nm for the second run (spectral acquisition in the range λ 200-400 nm). Peaks were identified on the basis of retention times, co-injections, and diode array spectral matching with standards. For the preparation of the calibration curve, standard stock solutions of compounds (1 mg/mL) were prepared in methanol and sonicated for fifteen minutes, filtered through 0.45 μm filters (Millipore), and appropriately diluted. Each of the plant extract/fractionate (10.0 mg/mL) were carefully weighed and prepared in methanol and was sonicated for 15-20 minutes, filtered through 0.45 μm filters (Millipore) before injection.

Cytotoxicity scavenging assays. To determine the effect of the polar extracts of *P. fulgens* on cell number over time, Sulphorhodamine B (SRB) assay [17] with slight modification were performed as described on various human cancer cell lines which include liver (Hep-2), leukemia (THP-1), lung (A-549), prostate (PC-3), Ovary(OVCAR-5) and CNS (SF-295) were seeded in flat-bottomed 96-well plates. The cells were incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2 adhere, and then media containing the extract at different concentrations were added. The plates were incubated for the next 48 h. The cells were fixed by adding 50 µL per well of ice-cold 50% TCA to each well for 60 min. The plates were washed five times in running tap water and stained with SRB reagent (0.4% w/v SRB in 1% acetic acid) 100 µL per well for 30 min. The plates were washed five times in 1% acetic acid and allowed to dry overnight. The final concentration of the DMSO (<0.2%, v/v) in the culture medium was maintained to avoid toxicity. SRB was solubilised with 100 µL per well 10 mM Tris-base, shaken for 5 min and the fluorescence was measured on automated 96-well Sunrise ™ Microplate Reader using excitation wavelength 570 nm and an emission wavelength 620nm. Cytotoxicity was expressed as Percentage growth inhibition of the cancer cell lines by the specified concentration of the plant extracts and standard drugs.

Statistical analysis. Data is presented as mean ± standard error mean (S.E.M.) of three independent determinations. One way analysis of variance (ANOVA) was used to compare the means.

RESULTS AND DISCUSSION

***In vitro* cytotoxicity assay screening of the polar extracts.** Among the three extracts used for the cell cytotoxicity assay, MeOH extract was found to be most potent and BuOH extract the least (Table-1). While the MeOH extract exhibited maximum cytotoxicity against HEP-2 cancer cell line (figure 1), DCM-MeOH extract showed significant activity against A-549 cancer cell line. BuOH extract showed no cytotoxic effect with the other cancer cell lines used for the study. The MeOH extract showed cytotoxic effects at higher concentrations (50 µg/mL & 100 µg/mL) against OVCAR-5, A-549, PC-3 and SF-295 cancer cell lines, while DCM-MeOH extract at 100 µg/mL exhibited significant cytotoxic effect against HEP-2 cancer cell line. The results are given in Table-1.

The most revealing facet of the antitumor properties studies was that the polar organics in the methanol and DCM-MeOH extracts are responsible for their activities, while the *n*-butanol extract have poor profile in each case. These observations led us to isolate and characterize the phenolic contents in those extracts of *Potentilla fulgens*. It may be noted that high concentration of phenolic compounds along with triterpenoids are already identified in other species of *Potentilla* [1, 4].

The *n*-butanol fraction of *P. fulgens* shows higher to phenolic content than methanol and dichloromethane-methanol extracts and than other polar extracts of the plant [4, 14]. The phenolic contents and levels in plants help to protect cells from various oxidative damage caused by free radicals and reduced the risk of other diseases in the body [6]. And from the HPLC analysis of the extracts, *n*-butanol extract was a choice for further examination for the phenolic constituents in *P. fulgens.*

Table 1: Percentage growth inhibition induced by polar extracts of *P.fulgens* in six human cancer cell lines at 10, 50 and 100 µg/ml
values are reported as mean ±SEM. All experiments were carried out in triplicates. The known anti-cancer drugs, 5-flurouracil and mitomycin-C were used as positive control

Cell Line Type →		THP-1	HEP-2	OVCAR-5	A-549	PC-3	SF-295
Tissue Type →		Leukemia	Liver	Ovary	Lung	Prostrate	CNS
Extract ↓	Conc. µg/mL	% Growth Inhibition					
	10	0 ± 3	30 ± 3	0 ± 3	0 ± 3	2 ± 3	0 ± 2
Methanol (MeOH)	50	0 ± 2	33 ± 2	4 ± 3	12 ± 2	1 ± 3	3 ± 2
	100	0 ± 3	63 ± 3	15 ± 2	24 ± 3	12 ± 1	11 ± 1
	10	0 ± 2	0 ± 2	0 ± 2	1 ± 2	1 ± 1	6 ± 1
Butanol (BuOH)	50	0 ± 2	5 ± 1	0 ± 2	1 ± 1	1 ± 2	0 ± 3
	100	0 ± 2	16 ± 3	0 ± 1	7 ± 3	1 ± 3	0 ± 3
	10	1 ± 1	0 ± 1	0 ± 1	5 ± 1	1 ± 3	2 ± 2
Dichloromethane: methanol (1:1) (DCM-MEOH	50	0 ± 1	8 ± 2	0 ± 2	29 ± 3	1 ± 2	2 ± 1
	100	0 ± 3	21 ± 1	10 ± 2	33 ± 2	1 ± 1	2 ± 3
Standard compounds:							
5-fluorouracil	20 µM	86 ± 3	-	75 ± 3	85 ± 1	77 ± 1	86 ± 1
Mitomycin-C	1 µM	-	85 ± 3	-	-	-	-

[a]*Percentage growth inhibition induced by polar extracts of P. fulgens in six human cancer cell lines at 10, 50 and 100 µg/mL. values are reported as mean ±SEM. All experiments were carried out in triplicates. The known anti-cancer drugs, 5-flurouracil and mitomycin-C were used as positive control.*

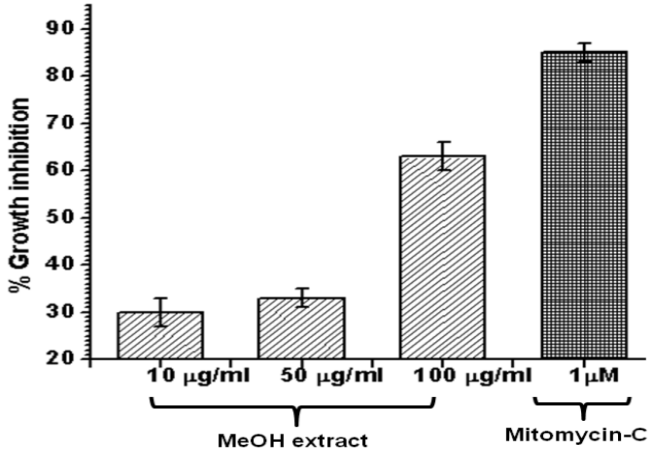

Figure 1. Percentage growth inhibition induced by MeOH extract of *P. fulgens* in HEP-2 cancer cell lines at 10, 50 and 100 µg/ml with reference to gold standard, mitomycin-C

The structures of isolated compounds (figure 2) were elucidated by using spectroscopic methods and were consistent with the reported data [15, 14].

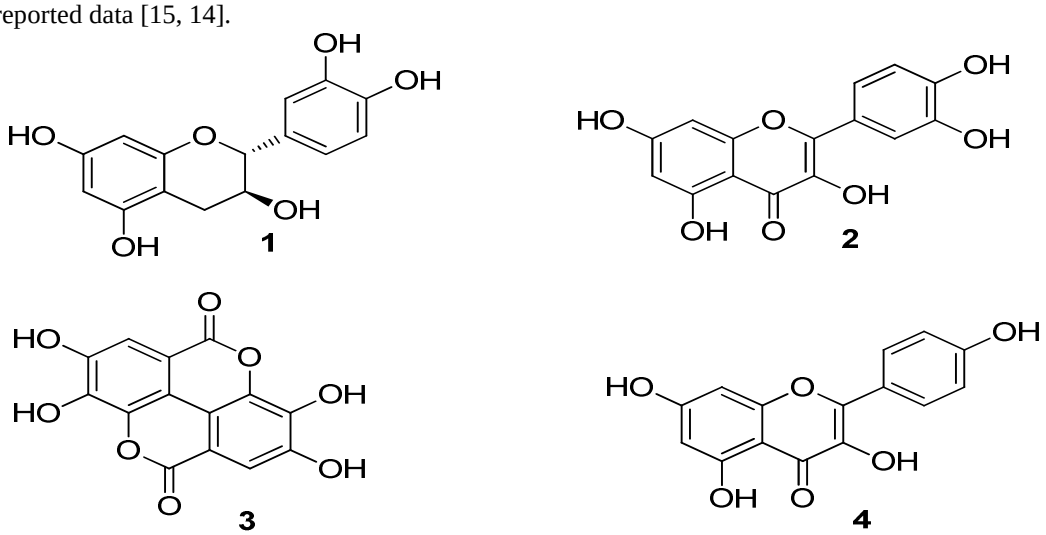

Figure 2. The structures of compounds 1-4 isolated from *P.fulgens* L

(+)-Catechin.The IR spectrum exhibited bands at 3403, 1613 and 1474 cm^{-1}. ^{1}HNMR (200 MHz, CD_3OD): δ 2.49 (dd, *J* = 8Hz, 16 Hz, H-4a), 2.84 (dd, *J* = 6 Hz, 16 Hz, H-4e), 3.97 (q,*J* = 7.7 Hz, H-3), 4.56 (d, *J* = 7Hz), 5.85 (d, *J* = 2Hz, 6-H), 5.92 (d, *J* = 2Hz, H-8), 6.69-6.83 (m, $3H_{2',5',6'}$). ^{13}CNMR (100 MHz, CD_3OD): δ 82.85 (C-2), 68.81(C-

3), 28.541(C-4),157.59 (C-5), 96.28 (C-6), 157.84 (C-7), 95.49 (C-8),156.92 (C-9), 132.20 (C-1′), 120.06 (C-2′), 116.09 (C-3′), 146.24 (C-4′), 146.26 (C-5′), 115.25 (C- 6′). TOF MS ES+ observed value $[M+H]^+$ 291.70.

Quercetin. The IR spectrum exhibited bands at 3374, 2640, 1606 and 1513 cm^{-1}.^{1}HNMR (400 MHz, CD_3OD): δ6.07 (d, *J* = 2Hz), 6.28 (d, *J* = 2 Hz), 6.7 (d, *J* = 8.8 Hz), 7.53 (dd, *J* = 2 Hz, 8.4 Hz), 7.6 (d, *J* = 2 Hz).^{13}CNMR (100 MHz, CD_3OD): δ 162.4 (C-2), 147 (C-3), 177.3 (C-4), 165.6 (C-5), 99.2 (C-6), 162.4 (C-7), 94.4 (C-8), 158.8 (C-9), 104.4 (C-10), 124.1(C-1′), 124.6 (C-2′), 116.2 (C-3′), 147.9 (C-4′),148.7 (C-5′), 115.9 (C-6′). ES+ observed value $[M]^+$ 302.

Ellagic acid. The IR spectrum exhibited bands at 3409, 2925, 1699 and 1116 cm^{-1}. ^{1}HNMR (400 MHz, CD_3OD): δ 7.5 (s). ^{13}CNMR (100 MHz, $CDOD_3$): δ 106.4, 110.1, 112.2, 136.2, 139.3, 147.9, and 158.9.

Kaempferol. The IR spectrum exhibited bands at 3303, 1965, 1600, 1520 and 1460 cm^{-1}. ^{1}HNMR (400 MHz, CD_3OD): δ 6.18 (d,*J* = 2Hz), 6.40 (d, *J* = 2Hz), 6.9 (d, *J* = 8.8 Hz), 8.09 (d, *J* = 8.8 Hz). ^{13}CNMR (100 MHz, CD_3OD): δ 160.5 (C-2), 148.05 (C-3), 177.3 (C-4), 165.6 (C-5), 99.29 (C-6), 162.7(C-7), 94.4 (C-8), 158.24 (C-9), 104.0 (C-10), 121.6 (C-1′), 123.7 (C-2′,C-6′), 115.9 (C-3′, C-5′),148.05 (C-4,). ES+ observed value $[M+H]^+$ 287.

A simple gradient elution-based RP-HPLC method was developed for the analysis and quantification of five major phenolics (1-5) in the polar extracts (figure 3 and 4).

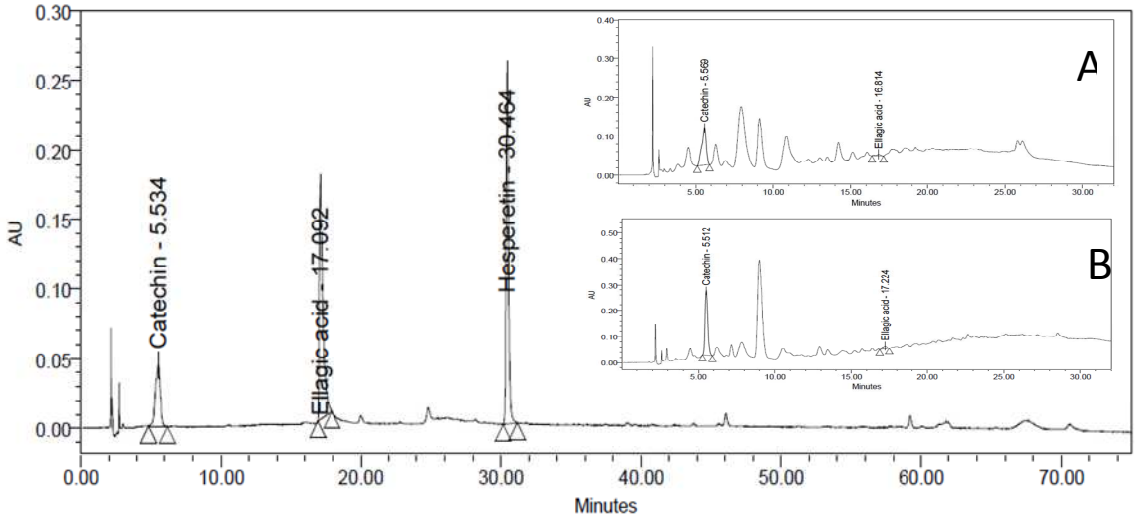

Figure 3. HPLC analysis at λ 280nm of Methanol Extract (A) and Butanol fraction (B)

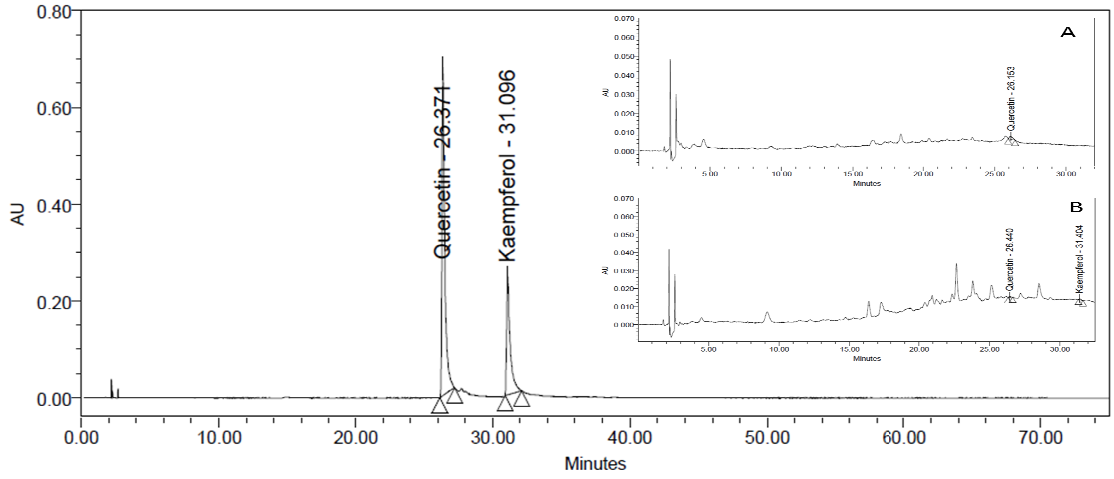

Figure 4. HPLC analysis at λ 380nmof Methanol extract (A) and Butanol fraction (B)

For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile and water with 5% of acetic acid was performed. The compounds were analyzed on the basis of their retention time (R_t) in comparison with the standard at two different wavelengths, *viz.* (+)-catechin (R_t=5.5), ellagic (R_t =17.09), hesperitin (R_t=30.4) at 280nm, while quercetin (R_t=26.3) and kaempferol (R_t =31.0) at 340nm. The

corresponding chromatograms of the butanol extract at 280 nm (fig. 1) and at 340 nm (fig. 2) indicate the presence of standard phenolic compounds **1**, **2**, **3** and **4**, which corresponded to (+)-catechin, quercetin, ellagic acid and kaempferol respectively. The chromatograms also indicate the presence of standard phenolic compounds **1**, **2** and **3** in the MeOH extract but not compound **4** (kaempferol). Though hesperitin was used as one of the standard compounds, it was not detected in either MeOH extract or BuOH fractionate. The (+) - catechin content was found to be higher than the other standard compounds in both the MeOH (11%) and *n*-butanol (17%) extracts (table 2).

Table 2: HPLC analysis of phenolics in extracts of P. *fulgens* L

Sample	Compound	R_t (in min)	Calibration R^2	% of content
	Catechin	5.56 ± 03	0.999392	11.0
	Ellagic acid	16.81 ± 0.06	0.999350	0.38
Methanol Extract	Quercetin	26.15±0.01	0.99998	3.98
	Kaempferol	ND	0.999898	ND
	Hesperitin	ND	0.999516	ND
	Catechin	5.512±0. 03	0.999392	17.04
	Ellagic acid	17.224 ±0.03	0.999350	1.11
Butanol Extract	Quercetin	26.440 ± 0.001	0.99998	0.48
	Kaempferol	31.404 ± 0.11	0.999898	0.45
	Hesperitin	ND	0.999516	ND
	Catechin	5.39±0.007	0.999392	0.62
	Ellagic acid	ND	0.999350	ND
DCM-MeOH Extract	Quercetin	ND	0.99998	ND
	Kaempferol	ND	0.999898	ND
	hesperitin	ND	0.999516	ND

ND= not detected. Amount represented in mg/g of the dry plant material [Mean±SEM]

Review of literature suggests that the compounds are reported for the first time in this plant which constitutes important bioactive phenolic compounds. No standard phenolic compounds, except (+)-catechin analyzed were identified in DCM-MeOH extract when subjected to HPLC analysis (figure 5).

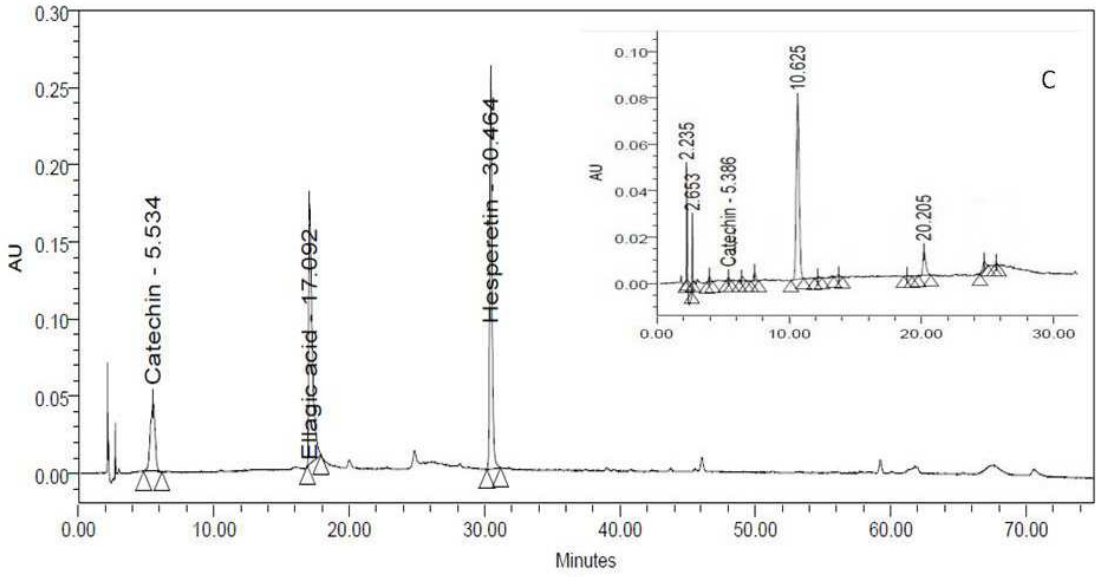

Figure 5. HPLC analysis at λ 280nmof DCM-MeOH Extract (C)

These results may help explain the pharmacological properties associated with this plant being locally consumed without any reports of the adverse effects [3]. The bioactive phenolics isolated from different sources have largely contributed to the activity shown in various extracts/or fractionate as these compounds are reported for various anti-oxidant, anti-carcinogens properties and, are also well known dietary compounds. (+)-catechin (**1**), quercetin (**2**), ellagic acid (**3**), and kaempferol (**4**) [figure 4] acts as potential antioxidative and cancer chemopreventive agents from plant-derived constituents apart from showing various biological activities. Quercetin (**2**) not only has antioxidant activity that enables it to scavenge active oxygen and electrophiles, but is known to down-regulate signal transduction in human breast carcinoma cells [1, 15]. Recently, it is has been reported to possess anti-complementary activity, a system which is responsible for adaptive immunity [14]. Since the dietary intake of flavonoids from different plant sources for the average human was estimated to be 16–25 mg/person [16]. *P. fulgens* may thus be added to the growing list of wild edible plants of the region and a source of polyphenols with beneficial properties.

In conclusion, our present study demonstrates *P. fulgens* L. as a source of potential anti-carcinogen agent. Collectively, these phenolic constituents would be expected to contribute to the overall cytotoxicity and antioxidant properties of the plant, although mechanism of action for the cytotoxic effect of *P. fulgens* extracts remains unclear and the anti-proliferation effect has not been reported. Detail studies is therefore, needed to delineate the relative contribution of this pathway to cytotoxicity. Our results suggest that the *P. fulgens* would be a potential candidate for the development of novel therapeutic agents to induce cell death in human carcinoma cells. The results obtained are promising and further studies are currently underway to determine the active principles and to understand its associated medicinal properties and further enhance utilization of the plant material as economic and available food source.

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