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COMPARATIVE STUDY OF THREE WILD EDIBLE FRUITS OF UTTRAKHAND FOR ANTIOXIDANT, ANTIPROLIFERATIVE ACTIVITIES AND POLYPHENOLIC COMPOSITION

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ABSTRACT

Ficus palmata, Pyrus pashia and *Ficus auriculata* are the popular wild edible fruits of Uttarakhand. The present study aimed to evaluate their total phenolic, flavonoid contents, antioxidant and antiproliferative activities by using two solvents namely 80% aqueous methanol and acetone. The results showed highest phenolic and flavonoid contents in the *Ficus palmata* acetone extract, while lowest in *Ficus auriculata* and *Pyrus pashia* methanol extracts respectively. Antioxidant activities were highest in the acetone extract of Ficus *palmata* and *Pyrus pashia* respectively. RP-HPLC analysis revealed abundance of gallic acid and catechin in all the fruit extracts, while traces of caffeic acid, coumaric acid and ellagic acid. All the fruit extracts possessed antiproliferative activities (22-63%) against cervical cancer cells C33A being highest in *Ficus palmata,* while showing no cytotoxicity to the normal peripheral blood mononuclear cells. Therefore, *Ficus palmata* fruit extracts could be used for the developing herbal formulation that can reduce the oxidative stress and prevent cancer development.

KEYWORDS: **Polyphenols, Antioxidant activity, Antiproliferative activity, RP-HPLC.**

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INTRODUCTION

Fruits are important sources of nutrition and medicines from the ancient times. They possess various bioactive substances that are responsible for their therapeutic properties in which, polyphenols hold great interest due to their redox potential that allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators[1,2]. These activities contribute in the multiple biological effects of polyphenols including anti-inflammatory, anti-allergic, antiviral, anti-aging and anti-carcinogenic activities and reduced risk of occurrences of cardiovascular diseases and cancers[3-6]. These health promoting properties of fruit phenolics led world wide great interest in the exploration of commercial and wild edible fruits for their polyphenol contents and their associated bioactivities[7-13]. *Ficus palmata (bedu), Ficus auriculata (timla)* and *Pyrus pashia (melu)* are among the most popular wild edible fruits of the Uttarakhand, India[14]. These fruits are of ethanomedicinal importance in addition to their great nutritional values. The fruits of *Ficus palmata* and *Pyrus pashia* are used in the treatment of diarrhea and dysentery[15,16]. *Ficus palmata* fruit is known to be useful in the lungs and the bladder diseases[17]. In contrary to the popularity of *Ficus palmata, Ficus auriculata* and *Pyrus pashia* fruits and the availability of numerous studies on its nutritional aspects[18], there is very limited literature on the polyphenol contents and their antioxidant, antiproliferative and antibacterial activities. Therefore, the present study was aimed to (1) assess the total phenolics and flavonoid contents in the fruit extracts of *Ficus palmata, Ficus auriculata* and *Pyrus pashia* using two different solvent systems; (2) to evaluate the fruit extracts for antioxidant activities using various biochemical assays; (3) determine the antiproliferative activities of the fruit extracts; (4) analyze the polyphenolic composition of fruit extracts.

MATERIAL AND METHODS

(i) Chemicals and reagents

All the chemicals were of analytical grade and more than 99% pure. 2,2-diphenyl-1-picrylhydrazyl (DPPH), catechin, nicotinamide adenine dinucleotide (NADH), phenyl methosulfate (PMS), nitro blue tetrazolium (NBT), β-carotene, linoleic acid and ferrozine were procured from Sigma–Aldrich (Steinheim, Germany). 2,2’-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was obtained from Calbiochem, Merck Company (Darmstadt, Germany). Other chemicals and reagents were purchased from HiMedia Pvt Ltd (Mumbai, India).

(ii) Collection, identification and authentication of the Ficus palmata, Ficus auriculata and Pyrus pashia fruits

Fresh ripe fruit samples (along with the small twig containing leaves) of *Ficus palmata, Ficus auriculata* and *Pyrus pashia*, were harvested from Narender Nagar locations of Tehri Garhwal, Uttarakhand, India. Fruits were cleaned under running tap water and kept at -20° C till use. The herbariums of the *Ficus palmata* and *Pyrus pashia* fruits were deposited to Systematic Botany Division, Forest Research Institute (FRI) while The herbariums of the *Ficus auriculata* fruits were deposited to Botanical Survey of India (BSI), Dehradun, Uttarakhand, India for botanical identification and authentication.

(iii) Preparation of extracts

Phenolic compounds were extracted using two different aqueous solvents namely 80% each of methanol and acetone[12]. Briefly, 25 g frozen fruits with seeds were homogenized in a mixer grinder for 5 min to make homogeneous slurry. 5 g fruit slurry was extracted thrice with 25 ml each of solvents for

30 min with constant stirring at room temperature (RT). The extracts were filtered, pooled and centrifuged to obtain the clear extracts. The clarified extracts were stored at -20°C prior to use within a month.

(iv) *Evaluation of total phenolics and total flavonoid contents*

Total phenolic contents were determined by the Folin-Ciocalteau method[19]. Each extract (0.1 ml) was mixed with Folin-Ciocalteau reagent (0.2 N, 2.5 ml) and allowed to stand at RT for 5 min. Thereafter, sodium carbonate solution (75 g/l in water, 2 ml) was added. Following incubation for 2 h, absorbance was measured using UV-Vis spectrophotometer (Model No. 119, Systronics, India) at 760 nm against water control. A standard calibration curve was also plotted using gallic acid (0-200 mg/l). The total phenolic contents were expressed as mg of gallic acid equivalent (GAE)/100g of frozen fruit weight (FW). Total flavonoid contents were determined according to Meda et al.[12]. Briefly, diluted extract (6.0 ml) was mixed with sodium nitrite solution (5%, 0.3 ml) and incubated for 5 min at RT. Afterwards, aluminium trichloride solution (10%, 0.6 ml) was added and incubated further for 5 min at RT. Absorbance of reaction mixtures were measured at 510 nm against water blank. A standard calibration curve of catechin (0.5 mg/ml) was plotted and the flavonoid contents were expressed as mg catechin equivalent (CE)/100g FW.

(v) Assessment of antioxidant activity

DPPH radical scavenging activity

The DPPH free radical scavenging activity was determined according to Singh et al[20]. Diluted extract (0.1 ml) was mixed with DPPH solution, (5 ml, 0.1 mM) and allowed to stand in dark at RT for 20 min. The control was prepared as above without any extract. Reduction in the absorbance of the control and samples was measured at 517 nm against water blank. The DPPH free radical scavenging activity was expressed as mg CE/ 100 g FW.

ABTS cation radical scavenging activity:

The ABTS cation radical scavenging activity was determined as described[21]. The diluted fruit extracts (50 µl) were allowed to react with fresh ABTS solution (3.0 ml) for 6 min, and then absorbance was measured at 734 nm. BHA was used as standard and the ABTS cation radical scavenging activity was expressed as mg BHA equivalent (BHAE)/100 g FW.

Ferric reducing power assay:

Ferric reducing power assay was performed according to Barreira et al[21]. Diluted extract was mixed with sodium phosphate buffer (2.5 ml, 200 mM, pH 6.6) and potassium ferricyanide solution (1% w/v, 2.5 ml) and incubated at 50°C for 20 min. Thereafter, TCA (2.5 ml, 10% w/v) was added and mixture was centrifuged, upper layer (5 ml) was removed, mixed with distilled water (5 ml) and ferric chloride solution (1 ml, 0.1% w/v). Subsequently, the absorbance was recorded at 700 nm and ferric reducing activity was expressed as mg of AAE/100g FW.

(vi) Inhibition of cancer cell proliferation:

Antiproliferative potentials of *Ficus palmata, Ficus auriculata* and *Pyrus pashia* fruits extracts were analyzed against two human cervical cancer cell lines namely C33A and HeLa cell lines (American Type Culture Collection, ATCC, Pune, India) and one non transformed Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were isolated from the blood of healthy individual and primary culture was established in RPMI 1640 medium supplemented with 10 % FBS. PBMCs were used as control to analyze the extracts induced cytotoxicity. C33A cells were maintained in Minimum Essential Medium (MEM) while, HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% antibiotic and antimycotic (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere with 5% CO_2 at 37°C. Cell concentration of $5x10^3$ in the respective

phenol free culture media were placed in each well of a 96 well flat bottom plate and allowed to grow for 24 h at 37°C in 5% CO_2. After incubation, the medium was removed and fresh media (100 µl) containing various concentrations of fruits extracts were added to the well. Control cultures received the extraction solution minus the fruits extract and blank contain 100 µl of growth medium with no cells. After the 48 h of further growth at 37°C in 5% CO_2, cell proliferations were determined using colorimetric MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide based cell titer 96 nonradioactive cell proliferation assay]. Percent cell proliferation was determined by measuring MTT absorbance at 450 nm (Fluostar Omega Spectrofluorometer, BMG Technologies, Offenburg, Germany). Each extract was checked at least in triplicate to determine the percent cell proliferation values.

(vii) Determination of polyphenolic contents through HPLC:

All Fruit extracts were analyzed for the presence of six phenolic acids namely gallic acid, caffeic acid, ellagic acid, coumarric acid, transcinnamic acid and tannic acid and two flavonoids such as catechin and myricetin by reverse-phase high performance liquid chromatography (RP-HPLC, Agilent Technologies HPLC system, model no 1120 compact LC). Extracts were dried at 40° in oven and re-dissolved in the methanol. Extracts were diluted up to five times in mobile phase (citrate buffer 0.001M and acetonitrile in the ratio of 70:30) and 20 µl of which was loaded on the C18 column packing with Inertsil ODS-3V (GL Sciences Inc., Japan, width and length 250 × 4.6 mm i.d. 5µm) equilibrated with mobile phase. The flow rate was kept constant to 1 ml min^{-1} in isocratic mode and the monitoring wavelength was 254 nm. The identification of each compound in the fruit extracts was based on the combination of retention time and spectral matching with the standard phenolic acid and flavonoid compounds

Statistical analysis:

To rule out any discrepancy, three independent extractions were performed. The results were expressed as mean of three independent experiments. Statistical analysis used the MS Excel software to calculate catechin, gallic acid, ascorbic acid, BHA, and EDTA equivalents, inhibition percentage, linear equations and correlation co-efficient While level of significant differences were determined by using non parametric ANOVA followed by Neuman-Keuls multiple comparison test using Prism 5 pad software.

RESULTS

Phenolic and flavonoid contents:

Total phenolic content of *Ficus palmata*, *Pyrus pashia* and *Ficus auriculata*, using two different solvent systems are presented in Figure 1. The total phenolic contents of methanol extracts varied from 58 to 490.5 mg GAE/100 g FW and that of acetone extracts were 74.51 to 494.7 mg GAE/100 g of FW using gallic acid as standard (R^2 = 0.9928). The highest amount of phenolic content was found in the acetone extract of *Ficus palmata* (494.7 mg GAE/100 g FW) while least amount was observed in methanol extract of *Ficus auriculata* (58 mg GAE/100 g FW). The total flavonoid content was determined using catechin as standard (R^2 = 0.9994) and it was varied between 96.45 to 201 mg GAE/100 g FW in methanol extracts while total flavonoid contents in Acetone extracts were 240 to 298 mg GAE/100 g FW. The highest amount of total flavonoids was observed in the acetone extract of *Ficus palmata* (298 mg GAE/100 g FW) while least amount was found in methanol extract of *Ficus auriculata* (96.45 mg GAE/100 g FW).

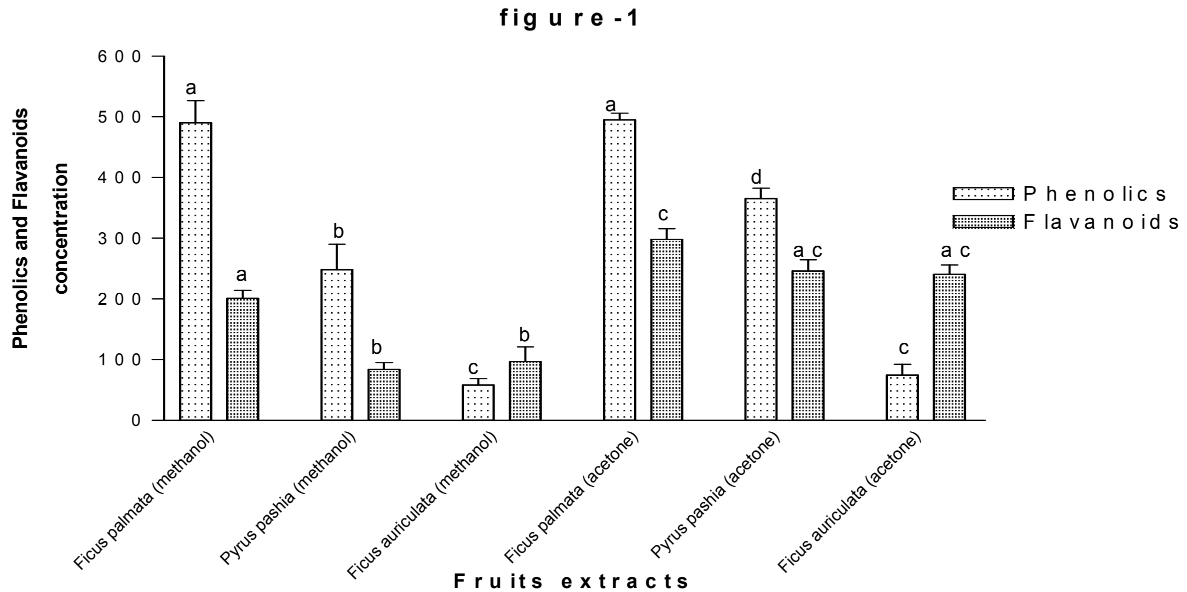

Figure 1

Total phenolics and flavanoids content of Ficus Palmata, Pyrus pashia and Ficus auriculata fruits extracts. Each value is expressed as mean ± standard error (n = 3). Means with different letters are significantly different (p<0.05).Total phenolic contents expressed as mg gallic acid equivalents per 100 g of Fruit weight while flavonoid contents expressed as mg Catechin equivalents per 100 g of Fruit weight. Each value is expressed as mean ± standard error (n = 3).

Antioxidant activity:

The antioxidant activities of three fruit extracts were determined using free radical scavenging activities and ferric reducing activities. The free radical scavenging activities were evaluated by DPPH and ABTS cation radical scavenging assays. The DPPH scavenging activity ranged 5.34 to 104.9 mg CE/100 g FW in various methanol fruit extracts (Table 1) while range of acetone extracts were 10.72 to 146.9 mg CE/100 g FW. It was highest in acetone extracts of *Ficus palmata* (146.9 mg CE/100g FW) and lowest in methanol extracts of *Ficus auriculata* (5.34 mg CE/100g FW) (Table 1). The order of DPPH free radical scavenging activity in three fruit is *Ficus palmata* >*Pyrus* pashia > *Ficus auriculata..* The ABTS assay measures the ability of fruit extracts to scavenge the cationic radical $ABTS^{\cdot+}$ produced by the oxidation of ABTS. The ABTS cation scavenging activity ranged from 27.59 to 557.09 mg BHAE /100 g FW in methanol extracts of different fruits while in acetone extracts; it was 32.77 to 729.45 mg BHAE /100 g FW. It was highest in acetone extracts of *Ficus palmata* (729.45 mg BHAE /100 g FW) and lowest in methanol extracts of *Ficus auriculata* (27.59 mg BHAE/100g FW) (Table 1). The order of ABTS free radical scavenging activity contents in three fruits was *Ficus palmate* >*Pyrus* pashia >*Ficus auriculata.* Ferric reducing activity was highest in acetone extracts of *Pyrus pashia* (758.8 mg AAE/100 g FW) while lowest in methanol extracts of *Ficus auriculata* (17.58 mg/100 g FW).

Table 1

Activities	Ficus Palmata (Methanol)	Pyrus pashia (Methanol)	Ficus auriculata (Methanol)	Ficus Palmata (Acetone)	Pyrus pashia (Acetone)	Ficus auriculata (Acetone)
DPPH (mg CE/100g FW)	104.29±14.01[a]	62.11±21.08[a]	5.34±1.19[b]	146.9±6.42[c]	96.15±8.46[a]	10.72±2.15[b]
ABTS (mg BHAE/100g FW)	557.09±31.86 [a]	493.7±24.58 [a]	27.59±7.64 [b]	729.45±18.10 [c]	546.73±15.32 [a]	32.77±5.91 [b]
Ferric reducing (mg AAE/100g FW)	77.6±12.26 [a]	526.02±6.27 [b]	17.58±5.6 [c]	146.67±15.77 [d]	758.82±26.45 [e]	19.2±4.32 [c]

Each value is expressed as mean ± standard error (n = 3).Means in rows with different letters are significantly different (p<0.05). DPPH scavenging activity expressed as mg Catechin equivalents per 100 g of Fruit weight. ABTS scavenging activity expressed as mg Butylated hydroxyanisole per 100 g of Fruit weight while Ferric reducing activity expressed as mg Ascorbic acid per 100 g of Fruit weight

Inhibition of cancer cell proliferation:

Antiproliferative activity of the fruit extracts were analyzed against cervical cancer cell lines namely C33A, HeLa and one normal PBM Cells using MTT assays. C33A and HeLa cells were cultured with an extract concentration equivalent to 0.667, 1.66, 3.33, 5.0 and 6.67 mg/ml of fruit while primary culture of PBMCs were incubated with 5.0 and 6.67 mg/ml fruit extracts. All the extracts demonstrated potent antiproliferative activity against C33A cells (~22-63%) (Figure 2). The IC50 value of all extracts varied from 5.19 to 14.64 mg/ml for C33A (Table 3). The extracts did not show antiproliferative activity against HeLa cells. Acetone extract of *Ficus palmata* showed highest antiproliferative activity (~63%) while it was low for methanol extract of *Ficus palmata* (~22%). The order of antiproliferative activity in the various fruits is *Ficus palmata* (63%) >*Pyrus pashia* (30%) > *Ficus auriculata* (31%) (Figure 2). None of the fruit extracts showed cytotoxicity to PBMCs.

Table 2

Quantity in different solvents (mg/100gm FW)	Gallic acid	Caffeic acid	P-Coumaric acid	Ellagic acid	Catechin
Ficus Palmata (Methanol)	196.31±1.57[a]	97.97±0.84 [a]	ND	2.29±0.157 [a]	117.16±2.61 [a]
Pyrus pashia (Methanol)	152.91±0.65 [b]	ND	ND	ND	24.76±0.66 [b]
Ficus auriculata (Methanol)	23.92±0.35 [c]	25.6±2.29 [b]	1.54±0.015	2.73±0.04 [a]	15.16±0.087 [c]
Ficus Palmata (Acetone)	220.25±2.49 [d]	ND	ND	5.72±0.5[b]	198±1.04 [d]
Pyrus pashia (Acetone)	192.16±0.90 [e]	4.37±0.13 [c]	ND	0.022±0.0003 [c]	156.96±1.49 [e]
Ficus auriculata (Acetone)	22.49±0.54 [c]	ND	ND	12.96±0.011 [d]	164.86±1.11 [f]

ND; Not Detected; Each phenolic and flavanoid contents expressed as mg per 100 g of Fruit weight. Each value is expressed as mean ± standard error (n = 3). Means in rows with different letters are significantly different (p<0.05).

Table 3

Ficus Palmata (Methanol)	Pyrus pashia (Methanol)	Ficus auriculata (Methanol)	Ficus Palmata (Acetone)	Pyrus pashia (Acetone)	Ficus auriculata (Acetone)
14.64	13.97	13.29	5.19	10.42	11.51

IC 50 value of Ficus Palmata, Pyrus pashia and Ficus auriculata fruits extracts against C33A cervical cancer cells.

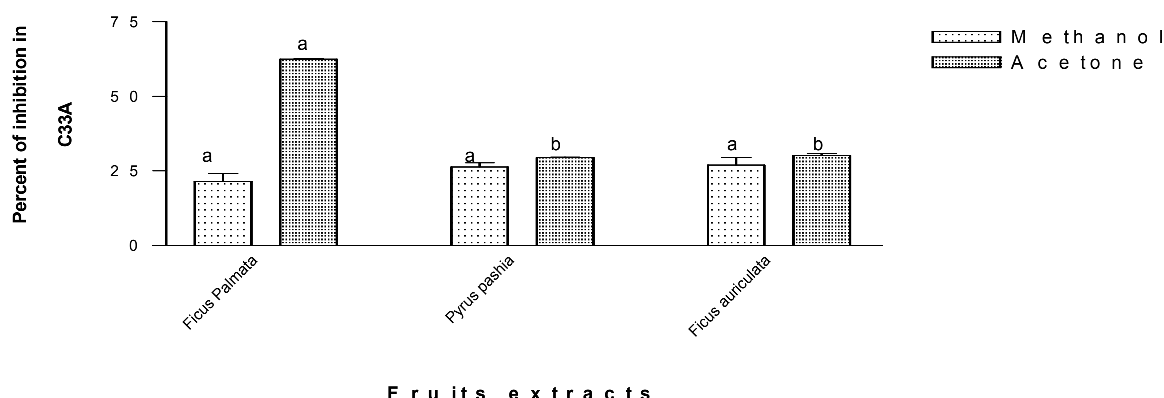

Figure 2

Antiproliferative activities of Ficus Palmata, Pyrus pashia and Ficus auriculata fruits extracts against C33A cervical cancer cells. Each value is expressed as mean ± standard error (n = 3). Means in rows with different letters are significantly different (p<0.05).

HPLC analysis of phenolic compounds:

The present method involving RP-HPLC analysis with C18 column (Inertsil ODS-3V stationary phase) was found to be simple, easy to use, reproducible and effective enough for the identification and quantification of major phenolic and flavonoid compounds in all the fruit extracts. Of the six phenolic acids (gallic acid, ellagic acid, caffeic acid, transcinnamic acid, p-coumaric acid and tannic acid) and two flavonoid compounds (catechin and myrecetin) tested for their presence. The phenolic acids and flavonoids contents were varied in all the fruits extracts (Table2). Gallic acid and catechin were commonly present in all the fruit extracts at high concentrations (gallic acid ranged 22.49 to 220.25 mg/100 gm FW; while catechin ranged 15.16 to 198 mg/100 gm FW for catechin). Ellagic acid was observed at significant level in acetone extracts of *Ficus palmata* (5.72 mg/100g FW) though it was also present in detectable level in *Pyrus pashia* and *Ficus auriculata*. Caffeic acid was observed at high level in methanol extracts of *Ficus Palmata* and *Ficus auriculata* while only acetone extracts of *Pyrus pashia* exhibited low but significant level. Courmaric acid was only observed in methanol extracts of *Ficus auriculata* that to in a very low level. However, myricetin and transcinnamic acid were found to be absent in all the three fruit extracts.

DISCUSSION

Present study was focused on the evaluation of the total phenolic and flavonoid contents in the 80 % acetone and methanol extracts of the three wild edible fruits namely *Ficus palmata, Pyrus pashia and Ficus auriculata* of the hilly region of Uttarakhand and the assessment of their antioxidant and antiproliferative properties. Among the three fruits, the order of phenolic and flavonoid contents and their antioxidant activities were *Ficus palmata* > *Pyrus pashia* > *Ficus auriculata.* The study

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showed higher recovery of phenolics and flavonoids in the acetone extracts of all the three fruits in comparison to that in methanol extracts. The free radical scavenging activities of *Ficus palmate*, *Pyrus pashia* and *Ficus auriculata* were also higher in acetone extracts than that in methanol extracts. Acetone exhibited highest recovery of not only phenolics and flavonoids, but also demonstrated higher antioxidant and antiproliferative activities suggesting acetone as the superior solvent system for the extraction of antioxidant and antiproliferative activities. Similar observations showing significant higher extraction of phenolics and flavonoids in the acetone extracts than in methanol extracts were earlier reported[12,10]. Among the three tested fruits, *Ficus palmata* showed highest phenolic, flavonoid, free radical scavenging activities, while *Pyrus pashia* showed highest ferric reducing activity. All the fruit extracts exhibited nearly similar antiproliferative activities against C33A cells (22~31%) however, the *Ficus palmata* acetone extract showed significantly higher antiproliferative activity against C33A cells (63%). The high anticancer effects of acetone extracts of *Ficus palmata* is supported by its relatively higher ellagic acid content as revealed by RP-HPLC analysis. Ellagic acid was earlier shown to possess antiproliferative activity against cervical cancer cells[22]. To the best of our knowledge present report is the first study showing the antiproliferative activities of *Ficus palmata, Pyrus pashia* and *Ficus auriculat* fruits. Our observations of phenolics and flavanoids content in the methanol extracts of *Ficus palmata* and *Pyrus pashia* are in consonance with the previous studies which showed high polyphenol contents[23]in these fruits. However, the present study reported much higher free radical scavenging and ferric reducing activities in the methanol extracts of *Ficus palmata* and *Pyrus pashia* in comparison to that reported by[23]. The present study further demonstrated presence of Gallic, Catechin and caffic acid in the fruit extracts of *Ficus palmata* and *Pyrus pashia* which were shown to be either absent or present in very low quantity in the previous study[23]. It is also possible that the fruit cultivars utilized by Saklani et al[23], might be different from the cultivar utilized in the present study in their climatic condition, harvesting and ripening stages and therefore their observations greatly differ from the present study. This fact could be also supported by the earlier studies which demonstrated that the polyphenolic composition and their antioxidant activities greatly varied according to cultivar, climatic conditions, altitude, harvesting periods and ripening stage of the fruits[24, 25].

CONCLUSION

In fruits, the highest phenolic and flavonoid contents and their antioxidant and antiproliferative activities were observed in *Ficus palmate* acetone extract followed by *Ficus auriculata* and *Pyrus pashia* methanol and acetone exracts. Therefore, it could be used for the development of the herbal formulation which could be consumed to reduced the oxidative stress and prevent cancer development.

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