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Antioxidant, Antidiabetic and Anticancer Effects of Persimmon Leave Extracts In vitro

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ABSTRACT

This study aims evaluate the antioxidant, antidiabetic and anticancer effects of persimmon leave extracts (PLEs). The results showed that all extracts had high amounts of phenolic and flavonoids components, the aqueous hot and cold extract exhibited the highest antioxidant activity determined by two methods (DPPH, ABTS), Vit. C and free sugar fractions determined by HPLC than ethanolic ex. Also, these extracts (aqueous hot and old) had high inhibition % of α- glucosidase and α- amylase enzymes that effect on diabetes and anticancer effect on two type of cancer cell the first was: $CaCo_2$ cell of colon and the hot ex have the highest inhibition % than other extracts, and the second was 549 cells of homo sapiens epithial adherent cancer cell and cold extract was the highest inhibition % against it. In this study we concluded that all aqueous extracts had higher effect as antioxidant, antidiabetic and anticancer than ethanolic.

1. Introduction

Persimmon *(Diospyros kaki*) belongs to the family of ebanaceae and has been cultivated over hundreds of years around Eastern Asia. The fruit of persimmon is eaten fresh or dry, whereas the leaf is generally used for tea due to their functional properties. In Japan, persimmon leaf is infused with hot water and drunk as a green tea (Kakinohacha) due to its healing effects in conditions of frostbite, paralysis, burns, as well as in stopping bleeding (WHO, 2001). The principal chemical constituents in the fresh or dried leaves of the oriental persimmon (Diospyros khaki) are flavonoids, triterpenes, and naphthoquinone, coumarins, tannins, sterols, organic acids, fatty acids, and volatile oil. Flavonoids, which have prospective therapeutic effects on the cardiovascular system as well as anti-atherosclerotic and anti-diabetic action, have been identified as the main bioactive components recovered from the ethyl acetate extract of persimmon leaves (EAPL) (Xi et al., 2015 and Zhou, et al., 2019). These polyphenols prevent the diabetes resulting from oxidative stress (Hosny and Rosazza 2002). According to estimates, 537 million persons worldwide were estimated to have diabetes mellitus which is leading to blindness, kidney failure, heart attacks, strokes and lower death rates by 3% between 2000 to 2019 in (WHO, 2021). The most prevalent form of diabetes is type 2 diabetes mellitus, which is characterized by carbohydrate, lipid, and protein metabolic abnormalities as well as errors in insulin production, nearly always with a significant role from insulin resistance (Kwon et al., 2005). These anomalies may result in pathologies such angiopathy, retinopathy, nephropathy, and other diseases. Due to its multiplex targets, plant-derived natural compounds are advantageous for reducing illness symptoms with fewer side effects (Zhang et al., 2007). Previous research has shown that an herbal prescription and plant-derived polysaccharides improved the body's immune system while preventing tumour development by increasing immune cell proliferation, speeding macrophage phagocytosis, and controlling cytokine production (Liu et al., 2013 and Zhang et al., 2013). Persimmon was shown to be effective in the treatment of prostate and breast cancers (Wang et al., 2002) oral carcinoma

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(Bei et al., 2005), the PLF dramatically increased thymus and spleen indices, serum interleukin-18 (IL-18) levels, monocyte/macrophage phagocytosis, serum hemolysin levels, and the activity of natural killer (NK) cells (Chen et al., 2018). The total flavonoids extract from the persimmon leaves may contribute to the antitumor and immunomodulatory activities of the ethyl acetate extract from the persimmon leaves. Recent studies reported in vitro proliferative and *in vivo* antitumor activities of flavonoids extracted from persimmon leaves against lung and prostate cancers (Chang et al., 2019). Human lymphoid leukemia cells (Takayuki 2005) and precancerous colon polyps in women (Fukuda and Shibata, 1994). The bioactive compounds in persimmon may also affect multidrug resistant (MDR) inhibiting activity. It enhances the accumulation of cancer cells due to the reduced activity of efflux pumps. MDR inhibitors from persimmon may help to treat nondurable cancer because of the modulating effects (Bei et al., 2005). The calyx (persimmon) extracts act as anticonvulsants and may alleviate the side effects of barbituric acid compounds (Itamurah et al., 2006). Physical, chemical, as well as microbiological stability have a significant role in determining the characteristics and quality of persimmon leaf kept by drying (Hossain et al., 2018a). The best method of drying in terms of maintaining color and nutritional content is freeze-drying. According to various drying methods, cultivars, and harvesting times, and no report has yet been produced on the bioactive components from Korean big persimmon leaves. based on drying techniques (freeze drying and hot air drying at 100°C for 30 minutes), the antioxidant capabilities of Korean persimmon leaves (Hossain et al., 2018b).

The aim of this study is to evaluate the antioxidant, anticancer and hypoglycemic effect of persimmon leaves with different methods of extraction.

2. Material and Methods

Material

Persimmon leaves (*Diospyros kaki L.* (Oriental persimmon) were harvested from the standing persimmon trees from the farm of Agricultural Research Center – Giza, between May and Juley 2021to kept into airtight plastic bags, and transported to the laboratory within 1h of harvest. The leaves were washed with tap water and dried in the shade for one week and ground into powder. All solvents and chemicals (ABTS, DPPH, methanol, ethanol) used were HPLC grades and obtained from Sigma Chemical Co. (USA).

Preparation of persimmon leave extracts (PLEs)

Preparation of aqueous extract (cold and hot)

The extraction was performed with, one volume of persimmon leaf powder was added to 10 volumes (1:10) of distilled water and extracted at room temperature at 90-100˚C for 3 h. The aqueous phase was filtered and concentrated with a vacuum evaporator (55˚C). Then lyophilization and the powder was stored at -4˚C until used (Hossain et al., 2018b)

Preparation of ethanolic extract

In the dark place for 24 hours, 50g of pulverized persimmon leaves were extracted with 1L of 80% ethanol for 24 hours. To create a crude ethanolic extract, the solvent was filtered, vacuum-evaporated at 40°C, and then frozen in the freeze-dryer system. Every preparatory step was carried out twice. Until it was used, the extract was kept at 4°C (Chang et al., 2019 and Zhou et al., 2019).

Methods

1. Assessment of total phenolic content using spectrophotometry to quantify the total phenolic content (TPC) according to (Chang et al., 2019), by Ciocalteu's Folin-phenol reagent At 735 nm, the absorbance was measured. Gallic acid was used to create a standard curve, and the results were expressed in milligrams of gallic acid equivalent (GAE) per gram of extract (dry weight, dw).

2- Assessment of total flavonoid content using the (Liu et al., 2009) spectrophotometry technique and the absorbance was measured at 510 nm. A standard curve was established using catechin, and the results were expressed as mg catechin equivalent

(CE)/100g extract (dw).

Antioxidant activity assay by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, the test is based on (Liu et al., 2009). The impact of DPPH free radical scavenging is reported as a percentage. The Inhibition concentration of 50% IC_{50} value represents the effective concentration capable of scavenging 50% of DPPH free radicals. The DPPH radical scavenging activity was calculated by equation (1). The IC_{50} value is the concentration of the sample required to scavenge 50% of the DPPH free radical.

(2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic (ABTS) radical scavenging activity). According to Olszowy and Dawidowicz, (2018) the spectrophotometric test of (ABTS) radical scavenging activity was performed. The control ingredient was ascorbic acid. The scavenging capability of the ABTS radical was calculated using the following equation (2):

Scavenging effect % = (1- Absorbance of sample at 517/Absorbance of control at 517) x 100 (1)

Inhibition%= -1 (Absorbance of sample –Absorbance of control) x100 (2)

3- Fractionation of phenolic and flavonoids components by using high performance liquid chromatography HPLC. To assess phenolic acids and flavonoids, leave samples were prepared according to the method specified by (Jakopic et al., 2009). Chromatograms were shown for phenolic compounds and flavonoids using wavelengths of 278 to 332 nm. Peak regions were used to identify and quantify all of the components according to method of (Schieber et al., 2001).

4- Fractionation of sugar by HPLC retention time criteria were used to detect sugars according to (Zelinski et al., 2014).

Vit. C determination by HPLC, samples were prepared according to the method described by (De Rosso et al., 2007). The chromatographic procedure used was based on isocratic method reported by (Lee, 2009).

Antidiabetic effect assay α-glucosidase inhibitor assay

The assay was carried out according to the method of (Abdallah et al., 2022), briefly, in 96-microwell plates, 25 µL of samples/blank were incubated for 10 min at 37C with 50 µL of *α*-glucosidase from *Saccharomyces cerevisiae* (0.6 U / mL) in phosphate buffer (0.1 M, pH 7). Then, 25 µL of 3 mM *p*NPG as a substrate in phosphate buffer (pH 7) was added, and the mixture was incubated again for 5 min at 37°C. Enzyme activity was determined by measuring the release of *p*-nitrophenol from the *p*NPG substrate at 405 nm using a microplate reader (Onega, USA). The % of inhibition of *α* -glucosidase was calculated according to the equation (3):

% Inhibition = [(Absorbance of blank – Absorbance of sample) / Absorbance of blank] x 100 (3)

In vitro α-amylase inhibitory activity

The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method according to (Wickramaratne et al., 2016). The extract was dissolved in minimum amount of 10% (Dimethyl sulfoxide) DMSO and was further dissolved in buffer ((Na_2HPO_4/NaH_2PO_4 (0.02 M), NaCl (0.006 M) at pH 6.9) to give concentrations ranging from 1.9 to 1000 µg/ml. A volume of 200 µl of α-amylase solution (2 units/ml) was mixed with 200 µl of the extract and was incubated for 10 min at 30°C.

Thereafter 200 µl of the starch solution (1% in water (w/v)) was added to each tube and incubated for 3 min/ 30 °C. The reaction was terminated by the addition of 200 µl DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) and was boiled for 10 min in a water bath at 85–90°C. The mixture was cooled to ambient temperature and was diluted with 5 ml of distilled water, and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer.

The blank with 100% enzyme activity was prepared by replacing the plant extract with 200 µl of buffer. A blank reaction was similarly prepared using the plant extract at each concentration in the absence of the enzyme solution. The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using the equation (4). The % α-amylase inhibition was plotted against the extract concentration and the IC_{50} values were obtained from the graph.

$$\%\ \alpha\ amylase\ inhibition = \frac{100 \times (Absorbance_{100\%}\ of\ control - Absorbance\ of\ sample}{Absorbance_{100\%}\ control} \quad (4)$$

Anticancer activity

Cytotoxicity on cells (MTT protocol) according to (Alley **et al**., 1988)

In vitro cell line Propagation

The RPMI-1640 medium used to grow the Caco2 cells included 10% inactivated fetal calf serum and 50g/ml gentamycin. A medium consisting of DMEM, 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50g/ml gentamycin was used to cultivate cells. All cells were maintained in a humidified 37°C, 5% CO2 incubator and subculture twice weekly.

Viability test for the analysis of cytotoxicity

Both the Caco2 and A549 tumor cell lines were grown in RPMI-1640 medium at 5x104 cells per well and 1x104 cells per well, respectively, in Corning 96-well tissue culture plates for 24 hours at 37 degrees Celsius in a humidified incubator containing 5% carbon dioxide. We took out the Caco2 cells or A549 cells media. A 1 percent crystal violet solution was poured into each well and left there for at least 30 minutes. All traces of the stain were taken away by rinsing the plates under running water. After adding and mixing 30% glacial acetic acid into each well, the absorbance of the plates was measured at 490 nm using a Microplate reader (TECAN, Inc.). To each well, including the untreated controls, we added 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) and then changed the media to 100 µl of fresh culture RPMI 1640 medium without phenol red. Incubation of the 96 well plates continued for another 4 hours at 37 degrees Celsius and 5% carbon dioxide. After taking an aliquot of the medium (85 µl), 50 µl of DMSO was added to each well, and the mixture was pipetted and incubated for 10 minutes at 37°C. An optical density microplate reader was used to take readings at 590 nm (SunRise, TECAN, Inc, USA). Cell viability percentages were calculated after the experiment was repeated three times. By means of the following Eq. Percentage of alive cells after OD treatment minus cells that were untreated after OD control = 100.

Graphical dose-response graphs for each conc. were generated in GraphPad Prism, and the CC_{50} and IC_{50}, the concentrations necessary to elicit harmful effects in 50% of intact cells, were calculated (San Diego, CA. USA)

Statistical Analysis

The collected data was analyzed using SPSS version 16 on a PC (Statistical Package for the Social Sciences). One-way ANOVA (Analysis of variance) tests using Duncan's multiple range test and p 0.05 was used to determine significance between various groups SPSS (1999) was used to examine the effects of different treatments.

3. Results and Discussion

Total phenolic and flavonoid contents in PLE

Recently, phenolic compounds have received a great deal of interest for their ability to regulate starch digestion and for their potent antioxidant properties. Their biological functions depend on their chemical structures, dosages, and duration of ingestion, and they are widely dispersed in plant diets. The family of phenolic chemicals include phenolic acids, flavonoids, and others. Flavonoids are the most extensively distributed category of phenolics in plants. Anthocyanin pigments, flavonols, flavones, flavanols, and isoflavones are flavonoids. Flavanols tend to polymerize into condensed tannins, while the majority of flavonoids exist as glycosides.

Particularly tannins, polyphenols have a strong antioxidant activity. Increased antioxidant levels in the body may defend against degenerative illnesses (Song et al., 2005). Flavonoids and isoflavonoids are one-electron donors in chemistry. These are conjugated ring structure and hydroxyl group derivatives that can act as antioxidants in cell culture in vitro or in cell-free conditions. Recent study suggests that the flavonoids present in persimmon leaves may contribute to persimmon's health benefits (Bei et al., 2009). According to the results in Table 1., hot water extraction is more successful than cold water extraction in removing the total phenolic components. There was 423 mg GA/g extract vs 224 mg GA/g extract in cold water, however ethanolic extract exhibited the lowest quantity of phenolic components at 175 mg GA/gm extract. In terms of total flavonoid content in PLE, ethanolic extract exhibited the highest concentration (622 mg CE/gm ex), followed by aqueous extract (622 mg CE/gm ex). (Yoo and Jeong 2009) evaluated crude persimmon leaf extract (PLE) and partially purified persimmon leaf extract (PPLE) (PPLE) The phenolic and flavonoid concentrations in PLE and PPLE were 230.019.6 mg/g and 475.538.7 mg/g, respectively, and 34.86.5 mg/g and 78.83.6 mg/g. Sun **et al.** (2011) discovered that TFPL extracts contained 1909.6 mg/g total flavonoids and reported that flavonoids are responsible for antioxidant action in late May and late June. (Hossain et al., 2018 a;b) employed a hot air drying method. Moreover, freeze -drying recovered 112.09 0.3 mg GAE/g d.m. and 84.21 0.61 mg GAE/g d.m. of phenolic compound in late May and late June, respectively. In comparison to other cultivars, the quantity of flavonoid content in late May (flowering) and late June (fruiting) was higher in cultivars treated by both drying methods. Using the hot air drying technique, the values were 37.83 0 and 34.17±0.83 (mg CE/g of d.m.) in late May and late June, respectively. Also, 36.55±0.54 and 34.44 0.96 (mg CE/g d.m.) of flavonoid content were found when blooming and fruiting phases were freeze-dried, respectively. No substantial differences amongst spices were detected. (Chang et al., 2019) determined that total phenolic content ranged (69.27 to 149.59 mg GAE/g extract) in different varieties of persimmon leaves harvested in September compared with October and November, and the same trend was observed in total flavonoids content in the same variety at the same harvest month, which ranged from 40.78 to 90.02 mg CE extract. (Grygoreiva et al., 2020) investigated the ethanolic extract of PL and discovered that it contained polyphenols at a concentration of 40 mg/g as gallic acid equivalent (GAE), total flavonoid at a concentration of 45 mg/g D.W as quercetin equivalent (QE), and total phenolic acid at a concentration of 5 mg/g D.W as Caffeic acid.

Table 1. Total phenolic and total flavonoids in persimmon leaves extracts (PLEs)

Treatment	Total phenolic mg GAE/100g as Gallic	Total flavonoids mg QE/100g as catachin
C	224.9^{b}±4.50	279^{b}±3.22
H	424.5^{a}±3.55	227^{c}±4.02
E	174.9^{c}±4.67	625.9^{a}±3.99

a,b,c significant differences between treatments ± standard division
C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

Phenolic components fraction in PLEs

Antioxidants components from plants preventing the peroxidation of lipids by the donation of a hydrogen atom from hydroxyl group attached to their chemical structure rapidly and form peroxyl radical (ROO) that ultimately leads to the formation of alkyl (aryl) hydroperoxide (ROOH), as shown in the following reaction: ROO− + PPH □→ ROOH + PP. The phenolic antioxidant (PPH) itself changes into polyphenol phenoxyl radical (PP), which

becomes stable after donating a hydrogen atom, by conversion into quinines, and it hinders the initiation of new chain reaction by reacting with another radical in which another phenoxyl radical is also included (Brewer, 2011 and Kawakami. et al., 2011). Using the HPLC technique for identifying ethanolic components on PLEs at 546nm in Table 2., seventeen components were identified, with ethanolic ex having the greatest phenolic concentration compared to aqueous cold and hot ex. Catachein (414.63 mg/100 g) was the most abundant constituent in etho ex, followed by salycillic acid, coumarin, ellagic acid, pyrogallol, and ferulic acid. The primary components of cold ex were pyrogallol (408.13 mg/g), catachein, salycillic acid, and coumarin. The latest sample was hot ex, and salycillic acid (172.21 mg/100g) was the primary component.

Table 2. Identification of phenolic compounds for persimmon leaves extracts (mg/100g)

Phenolic component	C	H	E
Pyrogallol	408.13	70.45	257.99
Gallic acid	28.48	12.36	19.317
3-Hydroxy Tyrosol	41.69	13.53	26.84
Catechol	14.53	2.30	6.21
4-Aminobenzoic acid	10.78	4.02	12.38
Catechein	209.98	126.38	414.63
Chlorogenic acid	116.01	62.37	141.41
Benzoic acid	10.38	8.54	ND
P-OH- benzoic acid	14.44	ND	27.97
Vanillic acid	43.20	14.91	38.29
caffeic acid	15.87	11.39	29.25
Caffeine	10.01	5.72	17.40
Ferulic acid	72.12	40.48	120.50
Salycillic acid	195.01	172.21	351.98
Ellagic acid	102.01	85.80	260.93
Coumarin	176.62	140.83	275.07
Rosmarinic	97.33	ND	93.07

C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

It is known that persimmon leaves contain large concentrations of flavonoids, which have distinctive UV-Vis absorbance at around 354 nm; hence, 354 nm was utilized as the primary acquisition wavelength in the HPLC-analysis. Eleven flavone and flavonol glycoside reference standards and the PLF extracts were examined using the HPLC technique, and their UV-Vis chromatograms at 354 nm are presented in the Table 3. The cold ex has the maximum flavonoids concentration, with rutin being the most abundant component at 6703.49 mg/100g, followed by hispiridin, apiening-6- arabinose-8- galactose, qurecetrin, rosmarinic acid and a little amount of author flavonoid. The second was ethanolic ex, with quercetrin 1252.44 mg/100g, apiening-6- arabinose-8- galactose, and rutin as the primary components.

The primary component of the third sample, the hot ex, was hispiridin with a concentration of 237.17 mg/100 g.

Flavonoid aglycones in persimmon leaves, in particular catechin, kaempferol, and quercetin, are said to have high antioxidant properties through acting as oxygen radical scavengers and metal chelators (Morel et al., 1993 and Birt et al., 2001). Flavonoids and isoflavonoids are one-electron donors in chemistry. They are conjugated ring structure and hydroxyl group derivatives with the potential to operate as antioxidants in cell culture *in vitro* or cell free settings. Previous research suggests that the flavonoids found in persimmon leaves may contribute to the health advantages linked to the fruit (Bei et al., 2005; Bei et al ., 2009 and Chen et al., 2018). (Chen et al., 2018) found myricitrin, rutin, myricetin, quercetin, and kaempferol in ethyl acetate extract of persimmon leaves by comparing retention periods and UV-Vis spectra. The five discovered flavone and flavonol glycosides comprised 96.9% of the total flavone and flavonol glycosides in the PLF, consisting of 60.7% rutin, 12.3% quercetin, 11.5% myricitrin, 9.3% kaempferol, and 3.1% myricetin, respectively. (Zhou et al., 2019) established and validated a method for evaluating the quality of the ethyl acetate extract from persimmon leaves (EAPL) and its commercial product, Naoxinqing (Brain and Heart Clear capsules). Based on HPLC–DAD-ESI-Q-TOF–MS analysis, myricetin-3-O-β-d-galactoside (1), myricetin-3-O-glucoside (2), quercetin- 3-O-β-d-galactoside (3), quercetin-3-O-β-d-glucoside (4), quercetin-3-O-(2″-O-galloyl-β-d-galactoside) (5), quercetin-3-O- (2″-O-galloyl-β-d-glucoside) (6), kaempferol-3-O-β-d-galactoside (7), kaempferol-3-O-β-d-glucoside (8), kaempferol-3-O-(2″-O-galloyl-β-d-galactoside) (9), kaempferol-3-O-(2″-O-galloyl-β-d-glucoside) (10), quercetin (11) and kaempferol (12). To find physiologically active chemicals, (Kown et al., 2021) separate as many components as possible from an ethanol extract of persimmon leaves. A novel flavonoid, kaempferol-3 -O-b-D-2"-coumaroylgalactoside (1), and a new natural compound, kaempferol-3-O-b-D-2"-feruloylglucoside (3), both exhibited antioxidative properties.

Table 3. Identification of phenolic and flavonoids for persimmon leaves extracts (PLEs) (mg/100g) C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

Flavonoid compounds	C	H	E
Apiening 6-arabinose 8-galactose	334.94	169.59	321.89
Hespirdin	1433.75	237.17	720.59
Rutin	6703.49	50.18	224.14
Apigenin 7-glucose	67.21	99.47	117.23
Quercetrin	278.40	156.80	1252.44
Naringin	6.68	2.743	15.69
Quercetin	24.53	10.92	41.63
Kampferol3-2-p-coumaroylglucose	58.30	47.65	135.92
Kampferol	16.57	ND	44.67
Apigenin	0.41	2.03	3.10

C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

Vit C content in PLEs

In this study we investigate the effect of different methods of extraction on the content of vit C. It was discovered that hot and cold extracts of persimmon leaves had a higher concentration of constituents than etho extract. In accordance with (Liang et al., 2000) in this study we confirmed that vitamin C is another major component of persimmon leaves.

Its concentration was discovered to be 13.9mg/g across the 410 types of leaves analysed (Michalska et al., 2017) discovered that freeze-dried samples had less vitamin C than other dried samples. It is likely that enhanced enzymatic activity during freeze-drying and thawing might result in ascorbic acid breakdown. Additionally, vitamin C retention may be negatively impacted by the longer drying time (72 hours) compared to hot-air drying (30 min). In this regard, hot-air drying is better than other methods for preserving the vitamin C content of persimmon leaves. After freeze drying, the vitamin C content may be graded as "Gabjubaekmok" >"Sangju-dungsi">"Sangam-dungsi"> Cheongdobansi" > "Suhong" In the blooming phase, vitamin C concentrations were greater than in the fruiting phase, ranging from 5 to 16 mg/g as ascorbic acid (Hossain et al., 2018b).

Antioxidant activity of PLEs

Among these compounds, the widely dispersed flavonoids in plants had the potential to scavenge free radicals (DPPH, ABTS). It is widely known that antioxidants may break the cycle of oxidative free radicals and generate stable free radicals that cannot begin or spread additional oxidation. The DPPH' radical was widely employed to test the antioxidants' ability to scavenge free radicals. Inhibition of lipid peroxidation is connected to the removal of the DPPH' radical. DPPH' radical involves a transfer of a hydrogen atom (Kaviarasan et al., 2007). According to the data shown in Table 4., aqueous hot ex has the strongest radical scavenging activity at varied concentrations (100, 200, and 300µg/ml), reaching 91.63%, compared to aqueous cold ex (88.90 %) and ethanolic ex (87.33%). IC_{50} was calculated and it ranged from 68.10 to 220 µg/ml. Due to the antioxidant capabilities of persimmon leaves, increasing the economic worth of this deciduous plant may be a viable option.

According to (Yoo and Jeong 2009) the radical-scavenging activities (IC_{50}) of crude persimmon leaf extract (PLE) and partly purified persimmon leaf extract (PPLE) were 23.83.2 ppm and 10.01.3 ppm, and 47.63.4 ppm and 22.43.3 ppm, respectively. utilizing 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radicals, as well as 5-lipoxygenase (5-LO) and cyclooxygenase (COX). PLE and PPLE inhibited 5-LO, COX-1, and COX-2 at IC_{50} concentrations of 77.111.7, 38.67.0, 47.47.4, 25.36.3, and 129.55.5, 84.52.3 ppm, respectively.

The PLE and PPLE had DPPH and superoxide radical-scavenging activity (IC_{50}) of 23.83.2 ppm and 10.01.3 ppm, and 47.63.4 ppm and 22.43.3 ppm, respectively. PLE and PPLE inhibited 5-lipoxygenase (5-LO) and cyclooxygenase (COX 5-LO, COX-1, and COX-2.

Sun et al. (2011) discovered that different concentrations of total flavonoid of persimmon leaf extract (TFPL) (12.5: 500 g/ml) inhibited antioxidant activity (14.48%, 21.15%, 32.06%, 51.28%, 68.73%, 75.62%, and 91.59% inhibition, respectively). Lower IC50 values are associated with higher DPPH radical scavenging activity. The outstanding antioxidant activity of TFPL on the DPPH' radical in this experiment may be directly related to its capacity to trap free radicals by donating a hydrogen atom. After harvesting by hot air and freeze-drying, 'Gabjubaekmok' persimmon leaf extracts had the highest percentage of DPPH (54.09 1.97 and 48.19 0.27%, p 0.05), whereas 'Cheongdobansi' leaf extracts had 41.11 1.25 and 35.65 0.69, respectively. DPPH levels in 'Sangju-dungsi' and 'Sangam-dungsi' leaf extracts were similar. Grygorieva et al. (2020) determined that the antioxidant activity of PLE (ethanolic) as determined by DPPH was 8 mg/g TEAC torolox comparable.

The second method ABTS free radical scavenging technique reveals that aqueous cold extract has the maximum scavenging activity 98.24% compared to aqueous hot and ethanolic extracts, respectively. Under same conditions, ABTS radical-scavenging activity was greater than DPPH radical-scavenging activity. (Lee et al., 2006) revealed that ABTS+ has a greater radical scavenging capacity than DPPH radical scavenging capacity of natural plant extracts. In both harvestings, 'Gabjubaekmok' persimmon leaf extracts had the greatest concentration of ABTS activity.

Table 4. Antioxidant activity of persimmon leaves extracts (PLEs)

Treatment	ABTS (%)	DPPH (%)	IC_{50} (µg/ml)
C	98.7^{a}±1.59	88.9^{b}±1.04	131.28
H	86.09^{b}±1.90	91.7^{a}±1.00	68.10
E	83.8^{c}±2.00	86.1^{c}±1.54	220

a,b,c significant differences between treatments ± standard division
C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

Fractionation of sugars in PLEs

Data illustrate in Table 5. evaluate the fractionation of sugar determined in PLEs with effect of different methods of extraction, the results showed that after hydrolysis with H_2SO_4 the Ethanolic extract has the highest quantities of sugars fraction, then aqueous cold extract and finally aqueous hot extract, arabinose was the main sugar in all extracts. In case of free sugars, it was found that aqueous cold ex had the highest quantities then aqueous hot ex and ethanolic ex the last one.

Table 5. Sugars fractions of persimmon leaves extracts (mg/100mg)

Sugar fraction	C	H	E
Free sugars			
Inulin	2196	1338.7	928.39
Stachyose	ND	ND	942.46
Sucrose	5419.4	18557.6	23192.3
Fructose	877.2	644.5	622.7
Arabinose	2537.2	1416.9	989.8
Galacturonic	1148.2	764.2	667.5
Sorbitol	ND	6.0	15.72
Ribose	5.74	ND	34.74
Hydrolysate sugar			
Fructose	13169.5	12948.5	14683.5
Arabinose	27261.5	24686.4	33633.1
Raffinose	1373.6	1887.6	1939.5
Galacturoinc acid	12078.4	10573.3	20917.6
Sorbitol	2177.74	ND	2715.3
Ribose	ND	3083.5	1968.4
Galactose	4977.4	10573.3	ND
Rhaminose	2196	3083.5	ND

C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

Antidiabetics' effect of PLEs

The inhibition of pancreas alpha-amylase (Ramasubbu et al., 2004 and Kawakami et al., 2010) and α-glucosidase (Kumar et al., 2011) could be one of major mechanisms responsible for the antidiabetic role of persimmon. So, the antidiabetic effects are dependent on degree of polymerization of bioactive components of persimmon. Therefore, inhibition of α-glucosidase and/or α-amylase by persimmon leaves extract may prolong overall digestion time, causing a delay in glucose absorption, consequently reducing the rapid increase of post-prandial blood glucose.

α-glucosidase inhibition

The *in vitro* inhibi-tory activity of PLE against yeast α-glucosidase is shown in Table 6. illustrate that PLEs were subjected to inhibition of α-glucosidase at concentration (100 to 1000 µg/ml) compared to acarbose as control and found that the aqueous cold ex of PLE was the highest inhibitory

effect with IC $_{50}$ 34.24µg/ml. These results related to high content of polyphenol components (especially flavonoid) the aqueous hot extract and ethanolic ex had IC $_{50}$ (34.11, 32.47 µg/ml) respectively. These resulted in agreement with (Sancheti et al., 2011) found that oral administration of persimmon leaf extract (methanol: water) (250 to 1000 mg/kg body wt.) considerably decreased blood glucose in normal and diabetic rats after glucose and maltose loading in a dose-dependent manner. These results propose that DKLE might show an antidiabetic effect by suppressing carbohydrate and glucose absorption from the intestine and can reduce the postprandial blood glucose rise. Also Bae et al. (2015) PLE suppressed glucosidase activity in a dose-dependent manner, at a dosage of 100 mg/ml, PLE reduced glucosidase activity by 70.5% and was 16.0% less powerful than the positive control, acarbose. (IC_{50}) for -glucosidase activity was 4.70 µg/ml. (El- Sayed et al., 2019) studied the effect of mulberry, persimmon leaves extract on inhibition α-glucosidase and found that administration of 1.2 g/Kg from mulberry, persimmon leaves and their blends represented 72.5%, 71.6% and 73.5% reduction in glucose levels, in rats administrated with water extract, and 73.1%, 68.4% and 71% reduction with rats administrated powder.

Table 6. α-glucosidase and α- amylase enzymes inhibition % of PLEs at concentration (1mg/ml)

Treatments	% inhibition α-glucosidase	IC_{50} (µg/mL)	α- amylase	IC $_{50}$ (µg/ml)
C	94.62^{a}±0.37	34.24 ± 1.39	60.6^{b}±1.03	278.9
H	92.76^{a}±1.8	34.11 ±1.91	74.5^{a}± 2.53	26.9
E	87.05^{b} ± 4.31	32.47±2.04	55.6^{c}± 2.23	387.9

a,b,c significant differences between treatments ± standard division
C (aqueous cold extract), H (aqueous hot extract), E (ethanolic extract)

α- amylase inhibition (%)

The suppression of carbohydrate-digesting enzymes is regarded a therapeutic approach for the treatment of type 2 diabetes (Tundis et al., 2010). The most crucial digestive enzyme is pancreatic alpha-amylase (EC 3.2.1.1), a calcium metalloenzyme that catalyses the hydrolysis of the alpha-1,4 glycosidic linkages of starch, amylose, amylopectin, glycogen, and various maltodextrins and is responsible for the majority of starch digestion in humans. A link between human pancreatic α -amylase (HPA) activity and the rise in postprandial glucose levels has been shown, highlighting the importance of inhibiting postprandial hyperglycemia (PPHG) in the treatment of type 2 diabetes (Watanabe et al., 1997). The potential of α-amylase enzyme inhibitors to prevent the digestion and absorption of dietary starch in the organism has led to their classification as starch blockers. To avoid the aberrant bacterial fermentation of undigested carbohydrates in the colon as a consequence of an excessive inhibition of this enzyme, which results in gas and diarrhoea, only a slight pancreatic α -amylase inhibitory activity is indicated (Watanabe et al., 1997).

Table 6. shows the activity of persimmon leaf extracts with different extraction methods against α -amylase enzyme as an indicator of the antidiabetic effect of persimmon leaf extracts (PLE). The inhibition% was evaluated with different concentrations (1.95, 3.9, 7.8, 15.62,31.25, 62.5, 125, 250, 500, 1000 µg/ml) to calculate IC_{50}. The results showed that aqueous hot extract has the highest inhibition% (74.5), aqueous cold extract was 60.6% then ethanolic extract 55.3 % with significant differences between treatments. It has been observed that the leaves of persimmon contain five flavonoid compounds: kaempferol 3-O-h-D-galactopyranoside (TR), kaempferol 3-O-h-Dglucopyranoside (AS), isorhamnetin 3-O-h-D-glucopyranoside (IS), quercetin 3-O-h-D-galactopyranoside (HY), and quercetin 3-O-h which have a great effect on this enzyme and low the diabetes (Saito et al., 1994 and Meng and Xu 1998).

The 50% inhibitory concentration (IC_{50}) for amylase, maltase, and sucrose are 1.7 µg/ml and 308 µg/ml, respectively, according to Tsujita (2016).

Anticancer activity

Using two types of human cancer cells, antitumor activity was evaluated of the persimmon leaf extracts (PLEs) *in vitro*. The concentrations of the extracts were (31.25, 62.5, 125,250,500, and 500µg/ml) and data showed that the toxicity% for the first type of cancer cells Caco2 cells colon was greatest in the aqueous hot extract with an IC_{50} of 95.74 g/ml, followed by the aqueous cold ex with an IC_{50} of 100.2 µg/ ml, and finally the ethanolic ex with an IC_{50} of 108.4 µg/ ml. Using the same dose of extracts, the second kind of cell was A549 cells of homo sapiens human lung epithelial adherent cancer cell. The findings revealed that aqueous cold ex exhibited the greatest percentage of toxicity with an IC_{50} of 181.18 g/ml, followed by ethanolic ex with an IC_{50} of 194.42 g/ml, and finally aqueous hot ex with an IC_{50} of 225.5 g/ml data presented in Fig. 1. In accordance with (Kawakami et al., 2011) treatment of A549 adenocarcinoma cells with PLE (persimmon leaf extract) and PLEg (200- galloly moiety) greatly improved the cytotoxicity of doxorubicin (DOX). In a dose-dependent manner, PLE and PLEg decreased the phosphorylation of check point proteins such as structural maintenance of chromosomes1, checkpointkinase1, and p53 in ataxiatelangiectasia mutants treated with DOX. PLE, and particularly PLEg, inhibited the G2/M checkpoint following DNA damage produced by DOX. In addition, (Wu et al., 2012) discovered that in an acute toxicity test, following pretreatment with water extract of persimmon leaves, the LD_{50} in male and female mice was more than 21.5g/kg (equivalent to 597.2g/kg in raw medicinal material), the ratio of polychromatic erythrocytes to normorchromatic erythrocytes (PCE/NCE) fell within the normal range at 10 g/kg (water extract), compared to 20 mg/kg for cyclophosphamide. The PLF in ethyl acetate extract inhibited the development of H22 liver tumours in mice (H22 hepatoma and S180 Sarcoma with a tumor inhibition rate of 50.5% and 38.2%, respectively), the PLF dramatically increased thymus and spleen indices, serum interleukin-18 (IL-18) levels, monocyte/macrophage phagocytosis, serum hemolysin levels, and the activity of natural killer (NK) cells (Chen et al., 2018). The total flavonoids extract from the persimmon leaves may contribute to the antitumor and immunomodulatory activities of the ethyl acetate extract from the persimmon leaves. Recent studies reported *in vitro* proliferative and in vivo antitumor activities of flavonoids extracted from persimmon leaves against lung and prostate cancers (Chang et al., 2019).

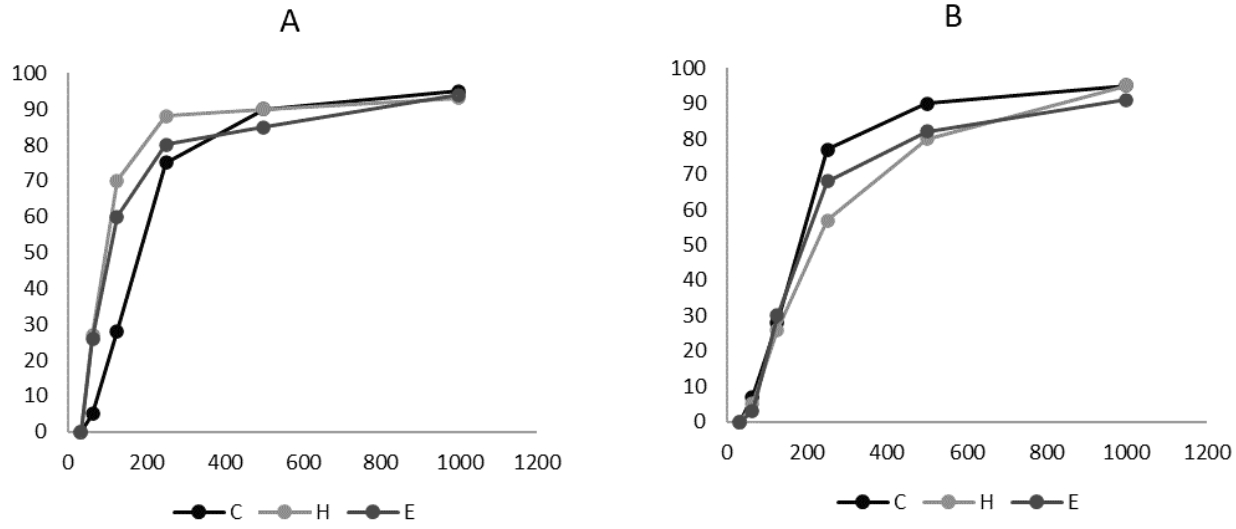

Figure 1. Anticancer effect (toxicity %) of PLEs (µm/ml) against two type of cancer cells. A: CaCO2 colon cell B: A 549 cells of homo sapiens human lung epithelial adherent cancer cells

4. Conclusion

The results showed that the aqueous extract (hot and cold) had the highest amount of phenolic component, Vit. C and free sugar fractions, antioxidant, antidiabetic effect and anticancer.

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