Biochemical analysis and comparative studies on *Diospyros kaki* extracts

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Graphical Abstract

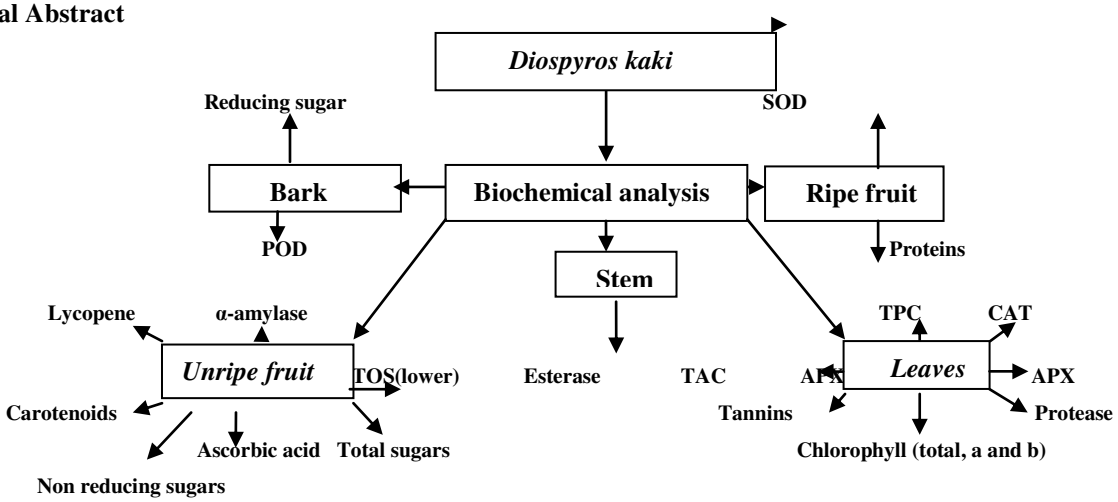

Fig1. Schematic diagram showing abundance of different biochemicals in different organs of *D. kaki*

Abstract

Background: Limited study on different organs of *Diospyros kaki* in biochemical figure of Pakistani plants still require considerable research activities due to its remarkable antioxidant properties.

Objective: This study was an effort to characterize antioxidant properties regarding *Diospyros kaki* with special reference to its detailed biochemical analysis and linked health claim.

Method: The biochemical analysis according to the role of certain bioactive molecules in different plant organs of *Diospyros kaki viz* enzymatic, non enzymatic antioxidants, hydrolytic enzymes and few other biochemical parameters such as total antioxidant activity, total oxidant status, total soluble sugars and protein along with pigments like chlorophyll (total, a and b), carotenoids and lycopene were studied.

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Results: Among enzymatic antioxidant, catalase and ascorbate peroxidase along with protease activity, total antioxidant capacity, total phenolic contents, tannins and pigments like chlorophyll (total, a and b) were observed highest in leaves. Unripe fruit showed significant α-amylase, ascorbic acid, non reducing sugars, total soluble sugars, total carotenoids, lycopene and significantly lowest total oxidant status. Similarly, remarkable highest amount of superoxide dismutase and proteins were predicted in ripe fruit whereas peroxidase activity and reducing sugars were investigated in bark. However, highest esterase activity was indicated in stem.

Conclusion: Our present findings concluded the fact that leaves, bark, stem, ripe and unripe fruit of *Diospyros kaki* could be exploited in pharmacology due to sufficient presence of different antioxidants but large number of antioxidants reflected in leaves and unripe fruit extract make them more potent and profound therauptic agents.

Future perspective: Keeping in mind the above facts and research findings, further pharmacological and biochemical explorations are needed to discover natural products.

Introduction

Plants are a rich source of different classes of bioactive secondary metabolites which are used for promoting public health or to combat various disorders (Céspedes et al. 2008; Jang et al. 2007). From the mid of 19th century, various bioactive chemical constituents have been reported from plants and many of them are being utilized as potent constituents of the present day medicine (Uddin et al. 2012). The important bioactive secondary metabolites are flavonoids, steroids, alkaloids, terpenes, coumarins, tannins and phenolic compounds for the medicinal purposes (Edeoga et al. 2005). These secondary metabolites are reported to reveal numerous biological activities including antimicrobial, antihypertensive, antioxidant,, anticancer activity (Savithramma et al. 2012). Medicinal plants do not play an effective role only in health sustaining and mitigating chronic ailments but as antioxidants also scavenge the oxidative damage that has been connected with more than one hundred diseases like diabetes, cardiovascular diseases, cardiac fibrosis, pancreatic liver diseases, atherosclerosis, joint disorders, neurological diseases amyotrophic lateral sclerosis, cancer, Huntington's disorder, Alzheimer's and Parkinson's ailments, age and related skin diseases (Hassan et al. 2017; Patil et al. 2009).

An appropriate balance is demanded between free radicals & antioxidants to keep biological activities normal and regular functioning. The biological sites can also be protected by quenching the free radicals with the potential use of antioxidants (Hassan et al. 2017). The human body has many internal components to snuff out free radicals; however, a significant contribution is by eating plants also. Plants include different kinds of natural products and most of them comprising to anti oxidative action (Narwal

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et al. 2014). The integrated antioxidant systems can balance the toxicity of oxidative reactive species which comprises enzymatic and non-enzymatic antioxidants A well organized antioxidative system in plant cell contains enzymatic antioxidants such as catalase ascorbate peroxidase, peroxidase and superoxide dismutase have the capability to quench free radicals, carry out H_2O_2 and intermediates of oxygen (Lee et al. 2007). Likewise, non enzymatic antioxidants are observed to remove excess reactive oxygen species (ROS) (Faize et al. 2011).

Currently, research on plants has been increased worldwide and big profile fact has attained to show use of medicinal plants on large scale in many traditional systems (Purane & Vidyadhara 2015). Amongst these**,** *Diospyros kaki*, which belongs to the *Ebenaceae* family is a deciduous small tree commonly known as Japanese fruit native to China and many other regions(Zhou et al. 2016). It is nutritious as well as medicinal plant enriched with many bioactive components like proteins, ascorbic acid (AA), lipids, sugar, vitamins, polyphenols (especially tannin), flavonoids, dietary fibers and minerals (Butt et al. 2015). Traditionally, this plant is used to cure different skin disorders such as skin eruptions, eczema and pimples (Kashif et al. 2017). In traditional medicine *Diospyros kaki* is used to treat diarrhea, arteriosclerosis, cough and apoplexy (Kim et al. 2009). *Diospyros kaki* is used as antitussive, sedative agent, carminative and to cure bronchial complaints in many traditional medicinal systems (Singh & Joshi 2011). Previously, it has been reported that *Diospyros kaki* contains many pharmacological activities including potent radical scavenging and antigen toxicity of seed (Jang et al. 2010) and anti-inflammatory action of leaves (Kim et al. 2016) and anti-carcinogenic, antihypertensive (Kawase et al. 2003) , anti-diabetic (Li et al. 2007) and antioxidant properties of peel and pulp(Jang et al. 2010). Different studies have been revealed about reported pharmacological activities and phyto-constituents profile on *Diospyros kaki* fruit in dermatology and cosmetics (Giordani et al. 2011; Xie et al. 2015).

In the current study, *Diospyros kaki* plant was chosen due to its promising and antioxidant status, however, limited or no information is available for potential use of its, stem, bark, leaves, ripe and unripe fruit. To our knowledge, this was the first reported work on detailed biochemical profile of different parts of *Diospyros kaki*. This study reflected the baseline information that could play a protective role for its possible applications in pharmaceuticals.

Materials and Methods

General information

For testing the antioxidant activity different organs including young green leaves, stem, unripe fruit and black bark of *Diospyros kaki* were collected from plant nursery, Lahore, Pakistan. In July to Sep, 2017, plant material was collected in plastic bags. To preserve quality the samples were stored in labelled

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bottles. Biochemical analyses were conducted at Plant Breeding and Genetics Division (MAB Lab-1) Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Sample extraction

Fresh plant samples (0.2 g) were carefully washed each alone under tap water and cut into tiny pieces and extracted in 2mL of KH_2PO_4 buffer (50mM) at pH 7.0. Plant organs at 4°C were centrifuged for ten minutes at 14,462 × g. For different biological analysis, supernatant was isolated. Three concordant readings were taken for all data.

Enzymatic antioxidants

Ascorbate peroxidase (APX) activity

Plant organs were homogenized for the determination of ascorbate peroxidise activity with composition of 0.05M KH_2PO_4 buffer having 7.0 pH. APX activity was described by the method (Dixit et al. 2001). Assay buffer was composed by mixing 0.5 M EDTA, 200 mM buffer with pH 7 and 10 mM ascorbic acid. APX activity was estimated by assay solution composed of 0.5 M EDTA, 10 mM ascorbic acid(AsA), 1 mL of H_2O_2, 200 mM potassium phosphate buffer nd 50 µL of supernatant. The estimation of oxidation rate of AsA was followed by decrease after every 30 s in absorbance at 290 nm (Chen & Asada 1989).

Peroxidase (POD) activity

Plant samples were mixed in a solution containing 0.1M EDTA, KH_2PO_4 buffer (50mM) at pH 7.0 and 1mM Dithiothreitol (DTT) for POD activity measurement. Activity of POD was estimated by (Chance & Maehly 1955) method with some modifications. For estimation of peroxidase activity, 200 mM guaiacol, 400 mM H_2O_2, 0.2 M phosphate buffer with7.0 pH, 545 µL distilled H_2O and enzyme extract (15µL) were mixed in solution. Reaction was started with the addition of the enzyme extract. At 470 nm the absorbance rise of the assay mixture was measured after every twenty seconds. A 0.01 min^{-1} change in absorbance is called one unit of POD activity. Sample weight was contributed to measure enzyme activity.

Superoxide dismutase (SOD) activity

Plant samples were mixed in a channel with compostion of EDTA (0.1 mM), 0.001M Dithiothreitol (DTT) and 0.05 mM KH_2PO_4 at pH 7 to estimate SOD activity by following (Dixit et al. 2001) method. The SOD activity was evaluated by estimating its inhibiting ability in photochemical reduction of (NBT)

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as described by method of (Giannopolitis & Ries 1977). Enzyme concentration responsible for inhibition of photochemical reduction of nitroblue tetrazolium upto 50% was considered as 1 unit of superoxide dismutase activity.

Non-enzymatic antioxidants

Total phenolic content (TPC)

For the estimation of phenolic compounds, a micro colorimetric procedure as descibed by (Ainsworth & Gillespie 2007) was followed with some modifications, which contained Folin-Ciocalteau (F-C) reagent. For evaluation, an ice cold pestle and mortar was used to homogenize 500 mg plant samples in 0.5ml ice cold methanol (95%). Then for incubation, samples were kept at room temperature for forty eight hours in the dark. Plant extracts were centrifuged at 14,462 × g for 5 min. Then supernatant was separated and used for TPC analysis. Mixture of supernatant (100µL) and 10% (v/v) Folin-Ciocalteau reagent (100 µL) was vortex vigorously and 700 mM sodium carbonate (800µL) was taken in all tubes and incubated for 1 hour at lab temperature. At 765 nm, Blank was used to auto zero the instrument before taking absorption of the assay mixtures. Afterwards, 0.1g of polyvinyl polypyrrolidone (PVPP) was mixed in above reaction mixture to detect tannin then centrifuge and again reading was measured at 765nm. With the help of different gallic acid concentrations a standard curve was drawn and a linear regression equation was measured. Using linear regression equation, phenolic contents of samples equivalent to gallic acid were determined.

Ascorbic acid (AsA)

2, 6-dichloroindophenol (DCIP) method (Hameed et al. 2005) was followed for measurement of ascorbic acid which measures reduced Vitamin C only. In short, each molecule of ascorbic acid converts a DCIP molecule into a reduced 2, 6-dichloroindophenol ($DCIPH_2$) and this conversion can be recorded as reduced absorption at 520 nm. The calibration curve was drawn with the help of known series of ascorbic acid concentrations. Ascorbate concentration in unknown sample was found by calculating simple linear regression equation.

Hydrolytic enzymes

Protease activity

Plant organs were extracted in 0.05 M KH_2PO_4 buffer with pH 7.8 to measure activity of protease. (Drapeau 1974) method was used to analyze protease activity followed by the casein digestion assay. According to this method, 1 unit of enzyme concentration was defined as release of acid soluble

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fragments equivalent to 0.001 A 280 min^{-1} with pH 7.8 at 37°C .On sample weight basis, enzyme activity was measured.

Esterase activity

According to the method of (Van Asperen 1962) α- and β-naphthyl acetate were used as substrates to determine α- and β-esterases. The reaction mixture possessed enzyme extract and substrate solution [1% acetone, 40 mM phosphate buffer with pH 7 and 0.03 M naphthyl acetates (α or β]. The assay solution was then kept in dark for incubation at 27°C for exactly 15 min and then added one mL staining solution containing one percent Fast blue BB and five percent sodium dodecyl sulfate with 2:5 ratio and incubated in dark at 27°C for 20 min. Absorbance at 590 nm was taken to calculate amount of alpha and beta naphthol obtained in µM/min/g of total sample was enzyme activity using standard.

Alpha amylase activity

A modified method as described by (Varavinit 2002) was followed to determine alpha amylase activity of plant sample.

Other biochemical parameters

Total oxidant status (TOS)

A formulated (Erel 2005) method was used to determine total oxidant status (TOS). In this assay the Fe^{+2}-odianisidine complex was oxidized to Fe^{+3} by oxidants present in the sample and measured ferric ion by xylenol orange (Harma et al. 2005). The assay solution possessed reagents R_1, R_2 and extract of sample. By using spectrophotometer, at 560 nm the absorption was calculated after 5 min. Hydrogen peroxide was used to prepare standard curve. The µM of H_2O_2 equivalents/ g were used to show results.

Total antioxidant capacity (TAC)

A modified method (Nenadis et al. 2007) was carried out for TAC analysis. Due to presence of antioxidants in sample, ABTS assay represents decrease of 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$ that is blue-green in color) into original ABTS (colorless compound). The antioxidants of the sample extract according to their content decolorize the $ABTS^{\bullet+}$ radical cation. The reaction mixture contained reagent R_1, sample extract and reagent R_2. After 5 min at wavelength of 660nm, the absorption of each reaction mixture was measured. This analysis used AsA (ascorbic acid) to develop a calibration curve. The results for antioxidant contents found in plant extracts were measured as µM AsA equivalent to1g.

Protein content

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Protein of plants were estimated by homogenizing samples in a channel with composition of 50 mM KH_2PO_4 buffer with pH 7.0. Previously described method (Bradford 1976) was executed for estimation of quantitative protein. 0.1 N sodium chloride and 5 µL supernatant were mixed in Bradford dye (1mL) for protein determination in samples. The reaction assay was allowed for five min to stand as protein dye complex. At 595 nm, absorbance was recorded with the help of UV-Vis spectrophotometer.

Reducing sugars (sugar content)

Dinitro-salicylic acid method (Miller 1959) was followed to determine the level of reducing sugars in samples. Phenol–H_2SO_4 reagent (Dubois et al. 1951) method was used to estimate total sugars. Difference is the non-reducing sugars.

Pigment Analysis

Pigments such as carotenoids, lycopene and total chlorophyll including (a and b) in 1500µL aqueous acetone were (80% (v/v) extracted, centrifuged for ten min at 14,000 rpm and subsequently supernatant was separated for the analysis as described by (LICHTENTHALER & Wellburn 1983) . The assay solution contained 1500 µL acetone and 500 µL extract of pigments to record absorbance at wavelengths of 663, 645, 505, 470, 453 respectively with UV-Vis spectrophotometer (NIAB-A (6) - SPH-006). The equations of (LICHTENTHALER & Wellburn 1983) were used to calculate concentrations (mg/g FW) of the pigments.

Statistical analysis

All the data was reported as mean ± SD. For analysis and organization of resulting data, descriptive statistics was applied. Two-way ANOVA with replications was used to analyze data. Significance of data was tested by analysis of variance and Tukey (HSD) Test at $p < 0.05$ and where applicable at $p < 0.01$ using XL-STAT software. Data was also subjected to principal component analysis using computer software Microsoft Excel along with XLSTAT Version 2012.1.02, Copyright Addinsoft 1995-2012 (http://www.xlstat.com).

Results and Discussion

Ripped *Diospyros kaki* fruit was reported to comprise 12.5 g/100g of total sugars (Piretti 1991) and 7.5 mg/100g of ascorbic acid (Ercisli et al. 2008; Özen et al. 2004). Similarly, lycopene in concentrations of 3.635 to 8.064 mg/100g (Vinha et al. 2011), 0.7 mg/100g [37] and 3.90 mg/100g (Homnava et al. 1990; Kondo et al. 2004) have been reported in ripped *Diospyros Kaki* fruits. In comparison, highest amount of total sugar (5.9 g/100g), ascorbic acid (vitamin C) (68 mg/100g) and lycopene (1020 mg/100g) was observed in unripe fruit in present study (Fig.1). So, unripe fruit can be a potent antioxidant due to higher amount of vitamin C and lycopene as well as comparatively lower sugar contents and resulted

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highest alpha amylase activity (49.43 mg/ g FW) and non reducing sugars (49.27 mg/g FW) in unripe fruit can have better therapeutic applications in diabetes. Amylase activity also shows a vital role in most of the physiological processes of the body (Giancarlo § et al. 2006). Amylase, the primary enzyme in the phenyl propanoid metabolism plays an important role in the composition of several defence-related secondary compounds like lignin and phenols (Hemm et al. 2004; Tahsili et al. 2014). Ascorbic acid reflects a significant and promising role in anti-aging (Tian-Hua Xu et al. 2012), skin whitening effects (Smith 1999; Traikovich 1999), skin rejuvenating agent (Crisan et al. 2015; Zahouani et al. 2002), anti-oxidant and free radical scavenger (Cathcart 1985; Erb et al. 2004). Lycopene has chemically shown to quench singlet oxygen and potent antioxidant ability in vitro (Conn et al. 1991; Di Mascio et al. 1989) to scavenge free radicals among naturally occurring carotenoids (Miller et al. 1996). Moreover, the significantly highest level of total carotenoids 32.48 mg/g FW and lowest total oxidant status 3383.33 µM/g measured as H_2O_2 equivalents were predicted in the unripe fruit of *Diospyros kaki* as compared to other organs except bark and leaves which were found almost statistically same (Fig 1). Precursors of vitamin A are some carotenoids present in *Diospyros kaki* (Nagao 2009) and also have anti-aging (Fusco et al. 2007) , immunoregulatory (Fraser & Bramley 2004) and antioxidant effects (MATHEWS-ROTH 1993).

The unripe fruit extract could be helpful for tissues growth and regulate body functioning as lower value of total oxidant status has been reported more potent in many biochemical processes (Mizrak et al. 2011). Similarly, resulted less amount of TOS in this work could reinforce the antioxidant capacity in unripe fruit for carotenoids,lycopene and other biochemical activities. Unripe fruit extract of *Diospyros kaki* has been reported as antioxidant, anti-diabetic, hypotensive, anticancer, anti-vininous, antif-ebril and demulcenis as well as juice from unripe fruit for treatment of hypertension(Singh & Joshi 2011) may reinforce our finding in unripe fruit amongst rest of other extracts.

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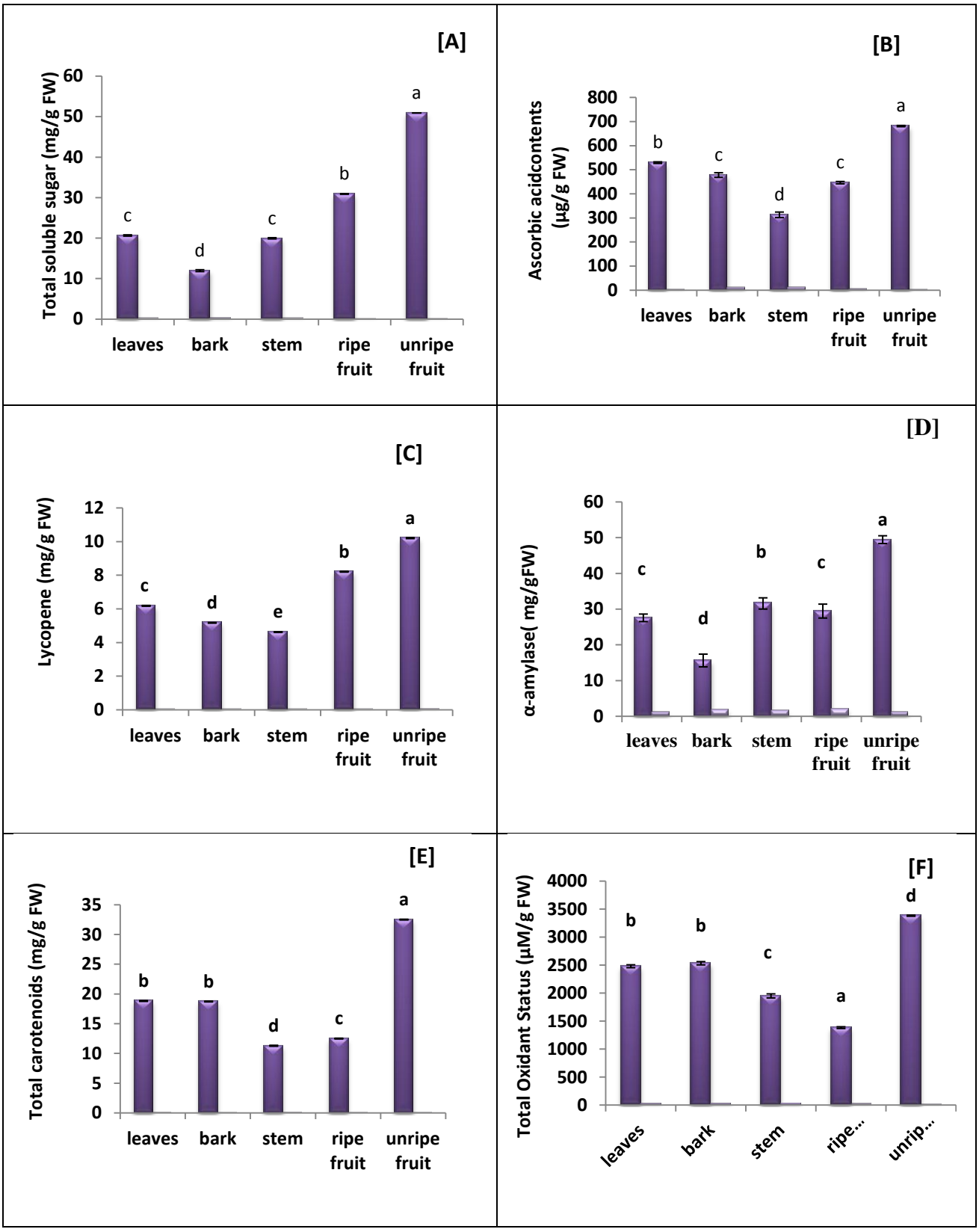

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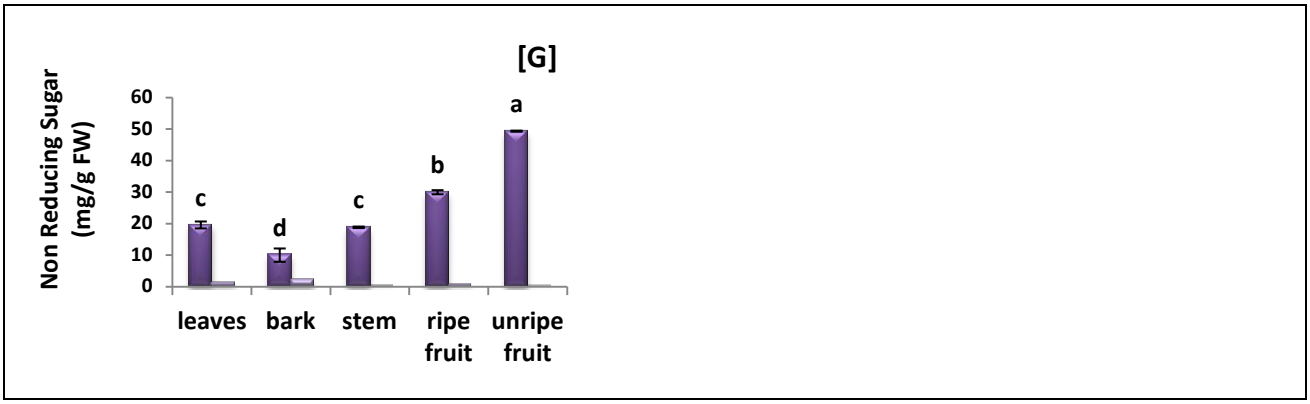

Fig.1. Comparison of total sugars (A), ascorbic Acid (B), lycopene (C), α-amylase (D), total carotenoids (E), total oxidant status (F) and non reducing sugars (G) in different organs of *Diospyros kaki.* Values are mean ± SD of three different experiments. Different letters on bars represent significant difference among different organs of *Diospyros kaki*

In this study, APX (706.7 units/ g FW) and catalase (4506.7 units/ g FW) were observed significantly highest in leaves amongst all other organs (Fig. 2). APX causes the detoxification of hydrogen peroxide (Hameed et al. 2014) while catalase with cofactor like Fe or Mn has the potential to degrade H_2O_2 into harmless water and oxygen (Khalid & Hameed 2017a) and has the capability to oxidize numerous toxins present in the body like alcohols, phenols, formic acid and formaldehyde (Karra-Chaabouni et al. 2003);(Hameed et al. 2014; Khalid & Hameed 2017b). Similarly, *Diospyros kaki* leaves also showed significantly highest (72183.33 µM/g FW) total phenolic contents (Fig. 2). Previously, TPC of *Diospyros kaki* fresh fruits or fruit juices have been studied and found strongest antioxidants among 62 analyzed fruits (Fu et al. 2011). Biological properties including endothelial function improvement, anti-inflammation, anti-ageing, anti-atherosclerosis, anti-apoptosis, cardiovascular protection and anti-carcinogen have also been possessed by phenolic components (Han et al. 2007) may further assist observed TPC in leaves during these research findings. Moreover, *D. kaki* leaves displayed highest amount (14150 µM/ g ±189.27) of tannins (Fig. 2). Previously, reported tannins in leaves found helpful in decreasing the chances of diabetes mellitus along with high potency to combat cancer (Lee et al. 2007; Park et al. 2002). They further show anti-allergic (Kotani et al. 2000), antibacterial (Kawase et al. 2003), lowering blood pressure (Jo et al. 2003) scavenging free radicals (Sakanaka et al. 2005), antioxidant, anti-mutagen (Achiwa et al. 1997) and anticancer activities (Gali et al. 1992). Likewise, intake of *Diospyros kaki* with tannin has been reported to contribute in prevention of hypercholesterolemia in humans and some animals (Gato et al. 2013). So, tannins reflected by *Diospyros kaki* leaves in current study could be contributed to above mentioned similar purposes. Total antioxidant capacity (TAC) detected by ABTS method in ascorbic acid equivalents was also found

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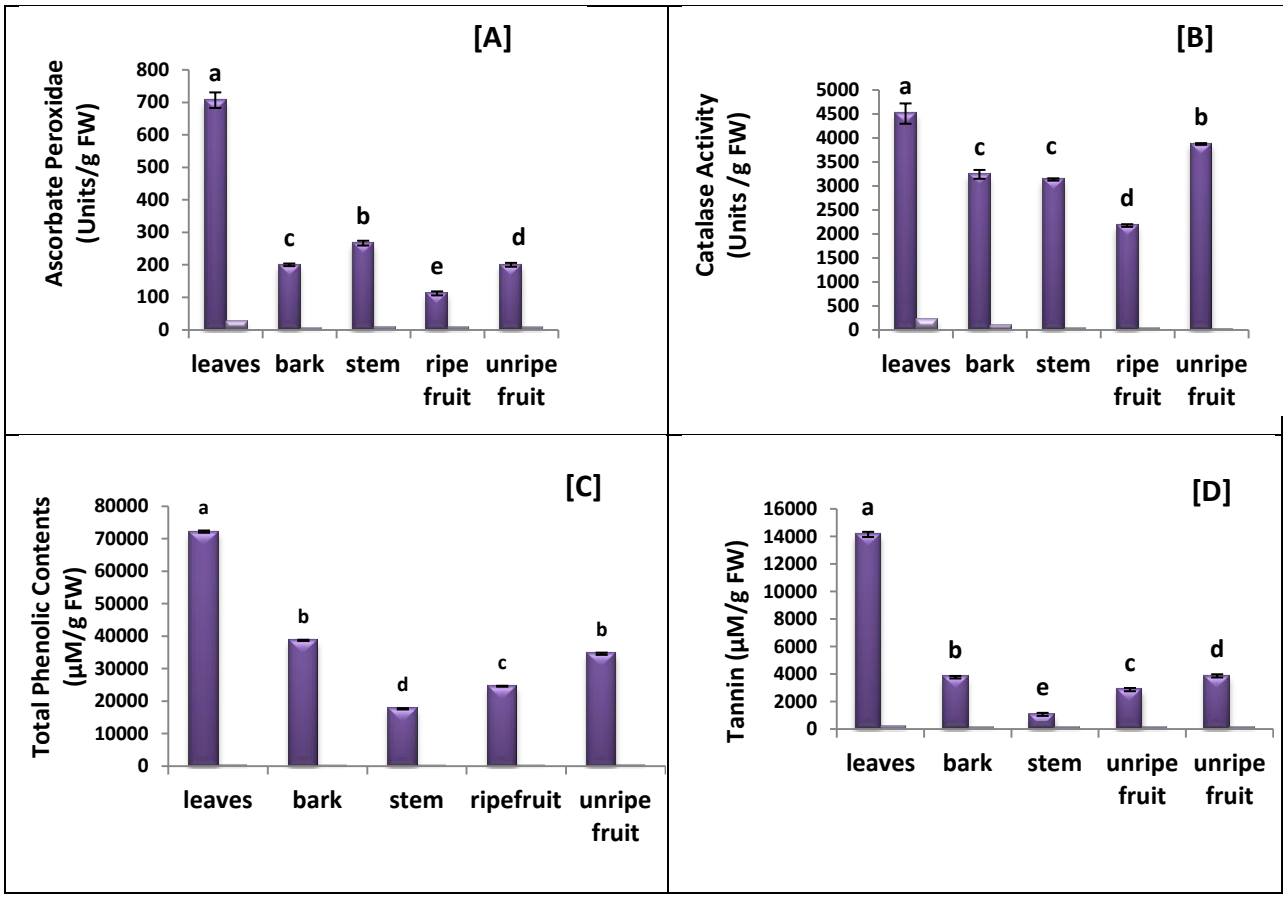
highest (15.7 ±1.15 µM/g.) in the leaves of *Diospyros kaki* followed by unripe fruit (14.9±0.66 µM/g) (Fig. 2). The defence system regarding TAC has been reported very effective in order to cure many diseases and improves immune system (Matés et al. 1999; Rubial et al. 1992). Moreover, observed highest value 412.34 µg/ g FW of total chlorophyll, chlorophyll a 187.6 µg/ g FW and chlorophyll b 224.8 µg/ g FW in leaves of *Diospyros kaki* (Fig. 2) may further enhance antioxidant potential of *Diospyros kaki* leaves in this study. Chlorophyll and its degradation products have been reported as important and available anti-inflammatory agents in abundance and promising for the improvement of phyto-medicine to heal inflammation and related disorders (Subramoniam et al. 2012). Chlorophyll molecules in pharmacy are used as photo sensitizer for cancer prevention(Mishra et al. 2012). So, *Diospyros kaki* leaves has also been found important in natural skin care due to their antioxidant potential (Mure et al. 2007).



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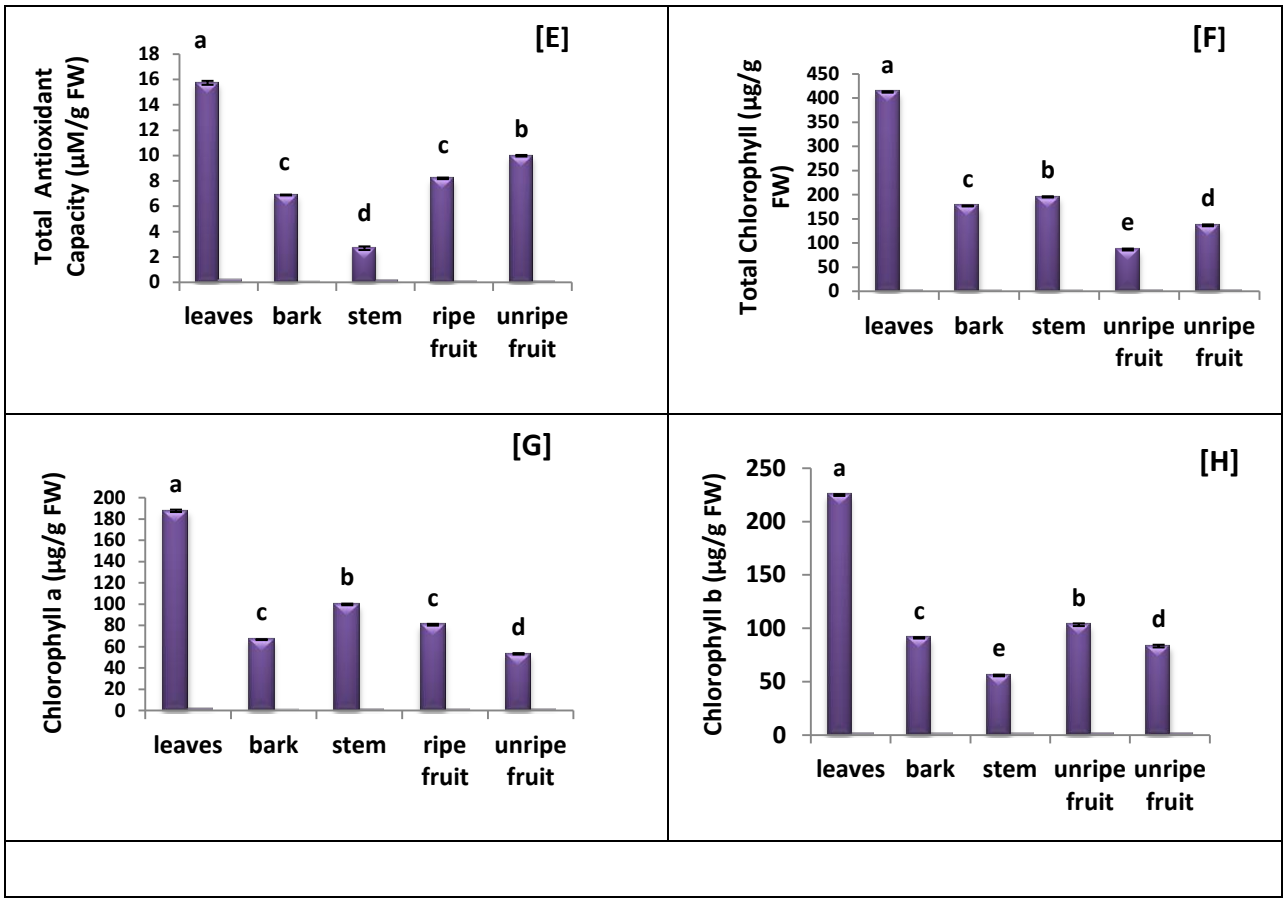

Fig. 2: Comparison of ascorbate peroxidase (A), catalase (B), total phenolic contents (C), tannins (D), total antioxidant capacity (E) ,total chlorophyll (F) and chlorophyll a (G) and chlorophyll b (H) in different organs of *Diospyros kaki.* Values are mean ± SD of three different experiments. Different letters on bars represent significant difference among different organs of *Diospyros. kaki.*

Total soluble proteins (TSPs) were detected to be highest (48.5±6.57g/100g) in ripe fruit of *Diospyros kaki* followed by unripe fruit (45.4.±5.47 g/100g) (Fig 3). Previously, unripe fruit of this plant have been reported to possess 0.58g/100g protein(Ercisli et al. 2008; Özen et al. 2004) while this study showed higher level of TSPs compared to earlier report. *Diospyros kaki* ripe fruit also displayed highest level (271.5 units/ g FW) of superoxide dismutase (SOD) that was significantly highest among other plant organs (Fig 3). SOD plays a key role being first internal antioxidant defence of the body. Various life-threatening disorders including stroke, heart attack atherosclerosis, chronic inflammatory conditions, disorders and various age-related acute produced by accumulation of oxidative stress can be significantly decreased in presence of enzymatic antioxidant (Maier & Chan 2002) could reinforce our findings in this study.

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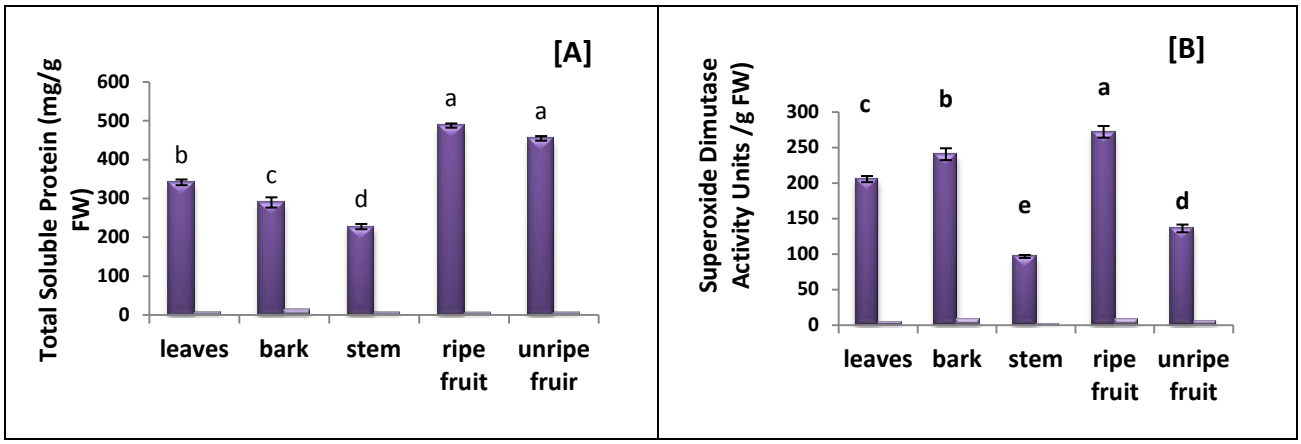
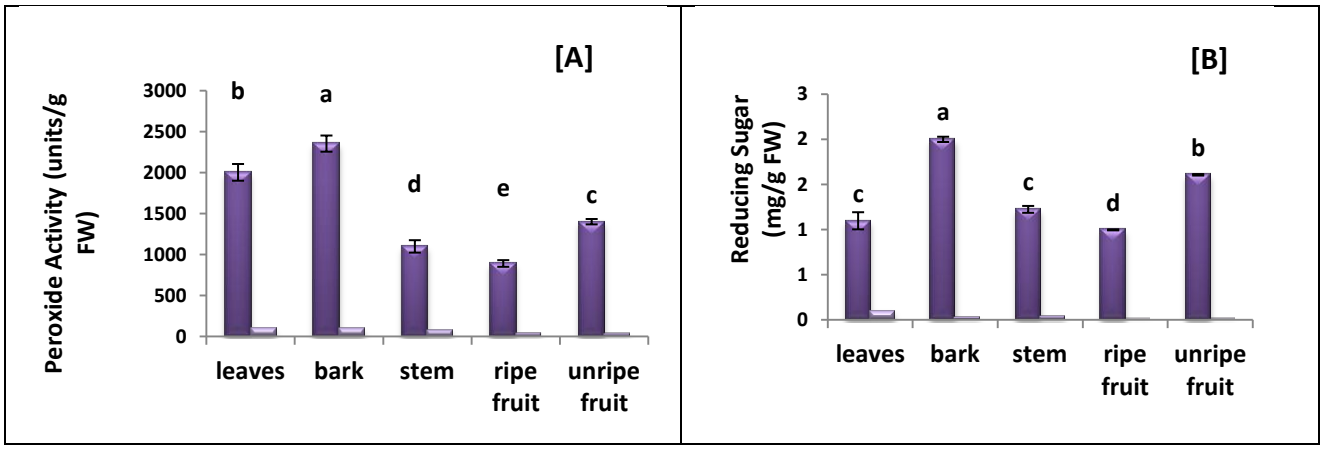


296 **Fig. 3**: Comparison of total soluble proteins (A) and superoxide dimutase (B) in different organs of
297 *Diospyros kaki.* Values are mean ± SD of three different experiments. Different letters on bars represent
298 significant difference among different organs of *D iospyros kaki.*

299 Reducing sugars (1.999 mg/g FW) and peroxidase (POD) (2353.20units/g FW) were found to be
300 significantly highest in bark of *Diospyros kaki* (Fig 4). Sugar contents have been reported to fight
301 against cytotoxicity of H_2O_2 to enhance cellular defenses in certain mammalian cells (Averillbates &
302 Przybytkowski 1994). Similarly, POD activity has capability to scavenge ROS (reactive oxygen species)
303 which causes cell oxidative injury (Vicuna 2005). As an ordinary skin-caring constituent in cosmetic
304 products, POD is also potentially used to remove hydrogen peroxide from the tissues (Maier & Chan
305 2002) supported our current findings in bark extract.



306 **Fig. 4**: Comparison of peroxidase (A) and amylase (B) in different organs of *Diospyros kaki.* Values
307 are mean ± SD of three different experiments. Different letters on bars represent significant difference
308 among different organs of *Diospyros kaki.*

309 The resulted highest level 11166.7 units/ g FW of protease in *D. kaki* stem (Fig. 5) can involve in the
310 degradation of several components of fungal pathogens. Protease involved in most of the physiological

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processes (Jadhav et al. 2017). Similarly, esterase 48.50 µM/g FW Esters can be converted into an alcohol and acid with the help of an esterase enzyme (Hou et al. 2012) .So, *D. kaki* stem may be used for above mentioned similar trials.

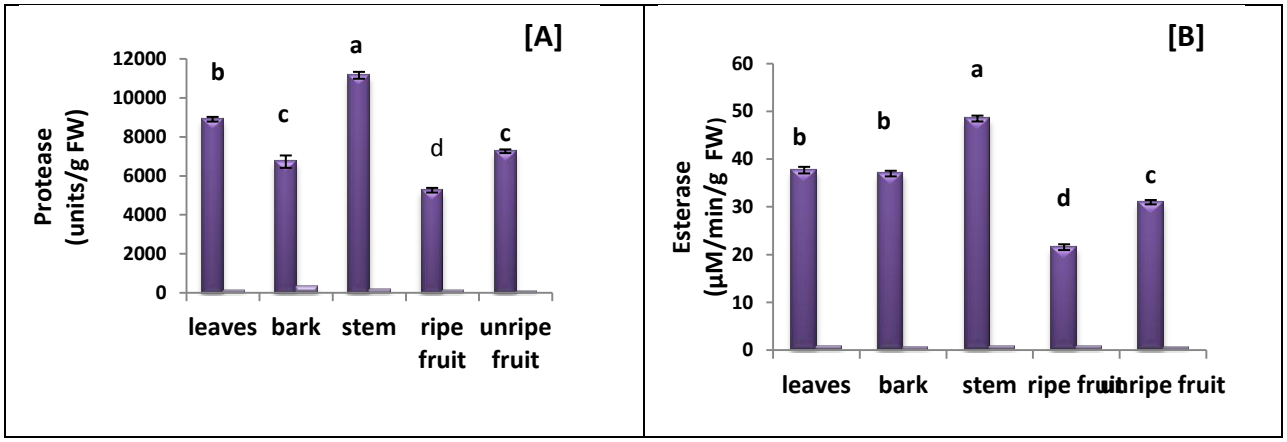

Fig. 5. Comparison of protease (A) and esterase (B) in different organs of *Diospyros kaki.* Values are mean ± SD of three different experiments. Different letters on bars represent significant difference among different organs of *Diospyros kaki.*

Conclusion

This study concluded the fact that leaves and unripe fruit extract due to abundance of antioxidants amongst rest of other organs have remarkable and profound antioxidant potential which could be capitalized in pharmaceutical herbal drugs for isolation, purification and characterization of new bioactive compounds. Based on comparatively, lower sugars contents and highest α-amylase activity unripe fruit of *Diospyros kaki* could be a potent anti-diabetic agent.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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