DETERMINATION OF ANTICANDIDAL AND ANTIOXIDANT POTENTIAL OF BLACKBERRY (*Rubus fruticosus*) FRUIT AND LEAVES EXTRACTS

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ABSTRACT: In the present study, water and methanol extracts of blackberry (*Rubus fruticosus*) were studied for antioxidant and anticandidal properties. The antioxidant properties of fruits and leaves were evaluated by determining radical scavenging power, lipid peroxidation inhibition activity and total phenol content measured with 1,1-diphenyl-2-picrylhydrazyl (DPPH) test, the thiocyanate method and the Folin method, respectively. Both water and methanol extracts have antioxidant potential. The water and methanol extracts of both fruits and leaves have antioxidant potential, ranging from 50.7 % to 64.4 %. Anticandidal activities of above extracts were also tested against clinical isolates of human pathogenic strains belonging 5 yeast species, Candida albicans, Candida glabrata, Candida parapsilosis, Candida krusei and Candida tropicalis by disk-diffusion method. The minimal inhibition concentration (MIC) value of each active extracts was also determined. The most effective antibacterial activity was expressed by methanol extract of blackberry leaves against with 15mm inhibition zone and 0.312 mg/ml MIC value.

***Index Terms*: Blackberry (*Rubus fruticosus*), Anticandidal activity, Antioxidat activity**

I. INTRODUCTION

R. fruticosus is a very prickly, scrambling, woody shrub with a perennial root system and biennial canes. It grows up to 2 m or more tall and is extremely variable in leaf shape and plant form. The fruit is an aggregated berry, 10-20 mm long, changing colour from green to red to black as it ripens, made up of approximately twenty to fifty single-seeded drupelets. Seeds are deeply and irregularly pitted, oval, coloured light to dark brown, and 2.6-3.7 mm long and 1.6-2.5 mm wide [1].

Among horticultural plants, berries have special importance due to their higher phytonutrients content. Their importance is accepted to increase further as the concept of "functional food" becomes more popular [2]. Rubus fruits are considered a healthy and nutritious food that contains valuable nutrients and nutraceutical compounds. Nutrients include vitamin C, B-vitamins, dietary fiber, α-tocopherol, tocotrienol, calcium, potassium, magnesium, carotenoids, linoleic acid and linolenic acid [3]. Bioactive compounds include phenolics such as ellagic acid and anthocyanins [4]. One of the most common components in functional food is of antioxidants. Antioxidants are substances that are able to delay the oxidation process by inhibiting the polymerization chain reaction initiated by free radicals and by preventing other substituent oxidizing reactions [5]. Free radicals are taken as a result of physiological activities, various external agents or nourishment and have roles in curing numerous chronic illnesses, notably cancer and aging [6]. Besides this harmful effects of free radicals are controlled by natural defense systems in body, that are necessarily supported by natural anti-oxidant components taken through diet [7].

Since ancient times, blackberries have been recognized for their uses in folk or herbal medicine. For example, blackberries were traditionally used as an anti-diarrheic and during pregnancy to shorten labor and to make it easier [8]. Modern studies have indeed shown that tea made from blackberry leaves can be effective for reducing the risk of dysentery and diarrhea [9]. Infusion from the leaves are traditionally used for easing childbirth-related muscle spasms, morning sickness, for colds,sour throats, diarrhoea, threat wounds, colic pain, uterin relaxant, etc.[10].

More recently, several studies were published on the antimicrobial effect of blackberry fruits [11-17].Despite the extensive studies on antioxidant and antimicrobial activity of several blackbeery species, to the best of our knowledge there have been no reports in the available literature that focus on the anticandidal activity of blackberry, especially on human clinical isolates. Therefore, attempts were made in this study to determine the anticandidal and antioxidant activity of blackberry fruit and leaves collected from Erzincan, Turkey.

II. MATERIALS AND METHODS

2.1.Plant material collection

The blackberry (*Rubus fruticosus*) fruits (at their optimum commercial maturity) and leaves were collected in Bayırbağ Town, Erzincan, Turkey. A voucher specimen number FEF 1651 was deposited in herbarium at Biology Department of Science and Art Faculty, University of Erzincan. The fresh fruit samples were packed on ice while being transported to the laboratory. Fruits samples were frozen at –20^{o}C until extraction.

2.2. Preparation of extracts

The leaves of blackberry were dried in shade and powdered with a blender. The fruits and powdered leaves were extracted with methanol in a Soxhlet apparatus for 24 h. Then methanol was evaporated with rotary evaporator. Water extracts were also prepared by adding boiling water to 20 g of powdered material in a glass flask and incubated at room temperature for 2 hours on a rotating shaker (200 rpm). Mixture was filtered using Whatman (No.1) filter paper and then filtrate was lyophilized. All extracts were stored in freezer at –24^{o}C until use.

2.3 Antioxidant Activities

***Antioxidant Activity*:** Briefly, stock extracts solutions were prepared at 2 mg/ml concentration. Required stock solutions were mixed with 2.5 ml of 0.02 M linoleic acid (Fluka) emulsion [contains an equal weight of Tween-20 (Sigma) in pH 7.4 phosphate-buffered saline (Sigma)], and the final volume was adjusted to 5 ml with phosphate-buffered saline (0.02 M, pH 7.4) in a test tube and incubated in darkness at 40 °C. Final concentrations of the extracts were 100 µg/ml. BHT (Sigma) was used as positive control (100µg/ml). The amount of peroxide was determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate after 24 hours incubation. Lower absorbance indicates higher antioxidant activity. To eliminate the solvent effect, the same amount of solvent used to prepare the solutions of test samples was added into the control test sample, which contains the linoleic acid emulsion. Measurements of antioxidant activity were carried out for three sample replications, and values are the average of three replicates. This activity is given as percent Lipid Peroxidation Inhibition which is calculated with the equation

$$\text{Lipid peroxidation inhibition (\%)} = \left[\frac{ControlAbs. - SampleAbs.}{ControlAbs.}\right] x100$$

DPPH Radical-Scavenging Activity : Briefly, 0.5 mM DPPH (Fluka) radical solution in methanol was prepared, and then 1 ml of this solution was mixed with 3 ml of the sample solution. Final concentrations of essential oils were 100 and 300 µg/ml. BHT was used as a positive control at the same concentrations. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. This activity is given as percent DPPH radical scavenging, which is calculated with the equation

$$Activity\,\% = \left[\frac{ControlAbs. - SampleAbs.}{ControlAbs.}\right] x100$$

Control contains 1 ml of DPPH solution mixed with 3 ml of ethanol. The measurements of DPPH radical scavenging activity were carried out for two sample replications, and values are an average of two replicates.

***Total Phenolic Compounds**:* Antioxidant compounds generally contain phenolic group(s). Because of this, amounts of phenolic compounds in each of the extract were compared to obtain more information about the extract(s) which possess(s) antioxidant potential. Briefly, extract solution was transferred into a tube and then final volume was adjusted to 4 ml by addition of distilled water. Afterward, 0.25 ml of Folin-Ciocalteu Reactive (FCR) (Fluka) was added into this mixture and after 3 min 0.75 ml of Na_2CO_3 solution was added. Subsequently, mixture was shaken on a shaker for 2h at room temperature and then absorbance was measured at 760 nm. Amount of total phenolic compounds were carried out for two sample replications, and values are an average of two replicates. Gallic acid was used as the standard for a calibration curve. The phenolic compound content was expressed as gallic acid equivalent using the following equation based on the calibration curve: Y=0,2582X Where Y is the absorbance of the sample and X is the gallic acid the equivalent (μgml^{-1})

2.4. Anticandidal Activity

Test Microorganisms: Anticandidal activity tests were carried out against clinical isolates of 36 *Candida* strains. Microorganisms were provided by Department of Clinical Microbiology, Medicine Faculty, Erzurum.

Disk-diffusion assay: The dried methanol and water extracts were dissolved in the extraction solvent (methanol and sterile distilled water). Final concentration was 30 mg/ml . Antimicrobial test were than carried out by disk-diffusion method . Using suspension containing 10^6 colony forming unit CFU/ml of yeast spread on nutrient agar (NA; Oxoid). The disk (6mm in diameter) were impregnated with extracts and placed on the inoculated NA. Negative controls were prepared using the same solvents (water and methanol) employed to obtain extracts. Amphotericin B (Sigma) for *Candida* spp. were used as positive controls. The inoculated plates were incubated at 35^{o}C for 48 h. Then anticandidal activity was evaluated by measuring the inhibition zone against test microorganisms.

Minimal Inhibition Concentration (MIC): The minimal inhibition concentration (MIC) values were also determined for the microorganisms, which were found to sensitive for the methanol and/or water extracts of fruits and leaves in disk-diffusion assay. MIC values of extracts against microbial strains were determined based on a micro-well dilution method. The inoculations of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Firstly, the extracts dissolved in 10% dimethyl sulfoxide (DMSO) were diluted to 10 mg/ml and then serial two fold dilutions were made in a concentration range (0.078–10 mg/ml) in a sterile test tube containing nutrient broth (NB). The 96-well plates were prepared by dispensing into each well 95 ml NB and 5 ml of the inoculums. A 100 ml of extracts initially prepared at the concentration of highest concentration was added the first well, then 100 ml from serial dilutions was transferred into other consecutive wells. The plates covered with a sterile plate sealer and then incubated for 48 h. The MIC was defined as the lowest concentration of the extracts to inhibit the growth of microorganisms.

2.5.Statistical Analysis

Statistical analysis were carried by using SPSS 15.0. Correlation analysis was carried out to Pearson correlation coefficient. Values at P<0,05 were considered to be significant and P<0,01 very significant.

III. RESULTS AND DISCUSSION

Antioxidant Activities

In this study, the antioxidant activity of water and methanol extracts of blackberry fruits and leaves we determined using the thiocyanate method, in which the quantity of peroxides formed in the emulsion during incubation is determined spectrophotometrically by measuring the absorbance at 500nm. The presence of lyophilized water or methanol extract at the 100 mg/mL concentration in the linoleic acid emulsion was able to reduce the formation of peroxides All extracts were able to inhibit lipid peroxidation, though no better than that of BHT (Figure 1). The most effective one was the methanol extract of blackberry leaf with 64,4% of inhibition. This was followed by the water extract of blackberry leaf of the with about 62,6% inhibition. The lowest activities were measured in both of the methanol and water extract of fruit with about 59,3% and 50,7% inhibition, respectively. In the light of these results (Fig.1) we could say that the leaf part of blackberry is more effective than blackberry fruit. However, we could also say methanol extraction is more effective than water extraction of blackberry.

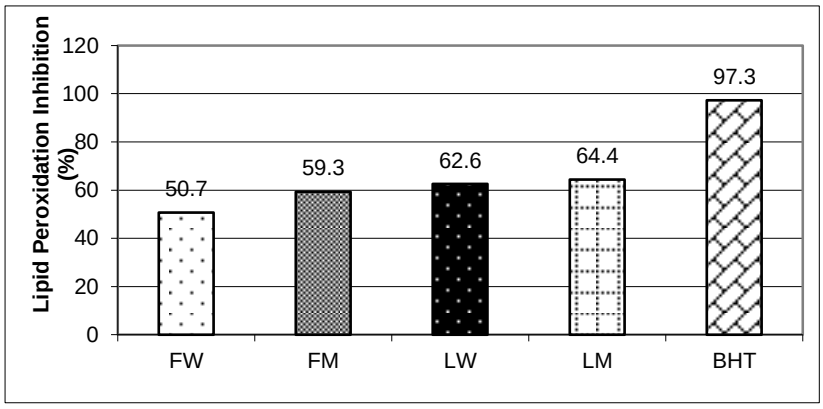

Figure 1. The Inhibition of Lipid Peroxidation by 100 µg/ml extract and BHT. Measurements were carried out after 24 hours incubation at 37^{o}C (L: Leaf, F: Fruit, M: Methanol extract, W: Water extract; BHT: Butylated hydroxytoluene)

As with inhibition of peroxide formation, all of the extracts exhibited DPPH radical-scavenging activity. The highest DPPH radical scavenging activity was measured in the methanol extract of the leaf with 92,2% scavenging, at the 300 µg/mL. This extract effectiveness was followed by leaf water extract (Fig 2). The less effective part of the blackberry, like peroxidation inhibition, was fruit. DPPH scavenging activities were higher in 300 µg/ml than 100 µg/ml leaf or fruit extracts. Also we have seen high activity better than BHT.

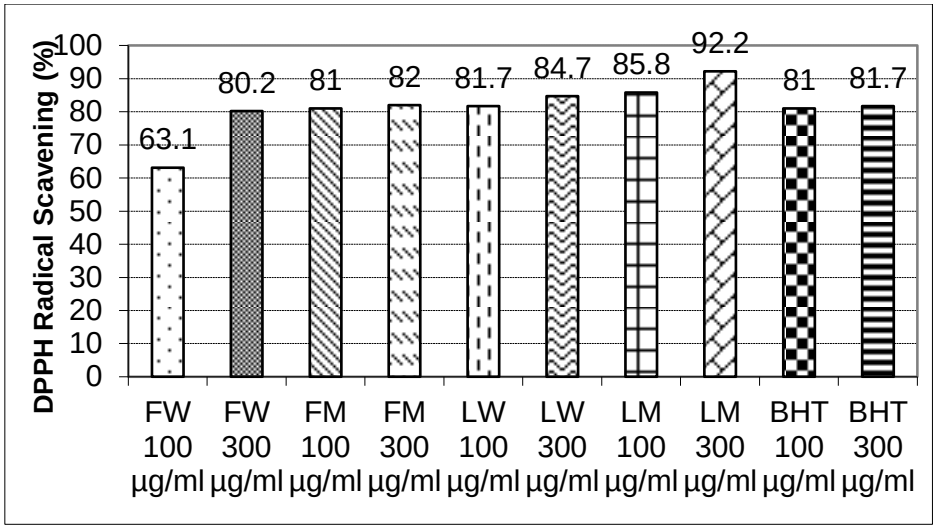

Figure 2. DDPH Radical Scavenging Activity (, L: Leaf, F: Fruit, M: Methanol extract, W: Water extract; BHT: Butylated hydroxytoluene).

In order to determine the amount of phenolic compounds present in the extracts, the absorbance of the extract solution was measured after incubating the samples with the Folin-Ciocalteu reagent. Like percent lipid peroxidation inhibition and DPPH radical scavenging activities, the contents of phenolic compounds of both water and methanol extracts of fruits were lower than leaf extracts (Figure 3).

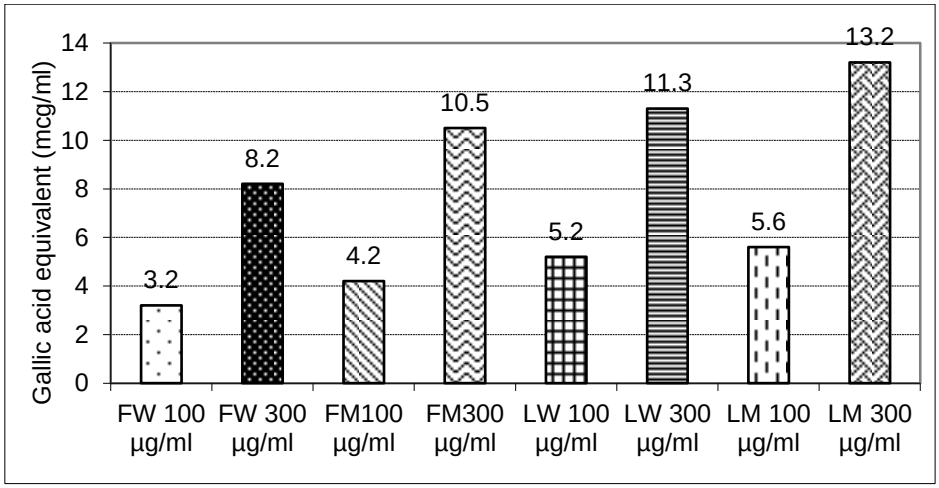

Figure 3. Amount of total phenolic compounds (L: Leaf, F: Fruit, M: Methanol extract, W: Water extract).

From the results given in figures 1, 2, 3, it is appear that there is a relation between each of the phenolic compounds amount, DPPH scavenging activity and lipid peroxidation activities. In fact according to Pearson correlation test there is statistically significant correlation between DPPH radical scavenging and phenolic compounds ($r = 0,911$; $p<.01$); between peroxide inhibition and phenolic compounds amount ($r = 0,924$; $p<.01$); between peroxide inhibition DPPH radical scavenging ($r = 0,902$; $p<.01$).

Anticandidal Activity

Anticandidal activities of methanol and water extracts of blackberry (*Rubus fruticosus*) were tested against 36 clinical isolates of *Candida* strains. The *Candida* species, isolation origins and strain numbers were given in Table 1.

Table 1. The *Candida* Species and Isolation Origins and Numbers

Microorganisms	Blood	Urine	Wound	Throat	Mouth	Total
Candida albicans	6	2	1	1	2	12
Candida glabrata	-	2	-	1	-	3
Candida krusei	2	-	1	1	-	4
Candida parapisilosis	3	3	-	-	1	7
Candida tropicalis	6	2	1	-	1	10
TOTAL						36

The anticandidal activity of water and methanol extracts of blackberry fruits and leaves were measured by the disc-diffusion method that is widely used for quick screening of natural products. Then minimal inhibition cconcentration (MIC) assays were used to determine the concentration at which the extracts were effective, according to the disc diffusion method.

Table 2 shows the anticandidal activities of methanol and water extracts of blackberry fruit and leaves against clinical isolates of human pathogenic yeast. The water and methanol extracts showed antimicrobial activity with inhibition zones ranging from 8 to 15mm. However, both methanol and water extracts of leaves exhibited similar characteristics in their activity against *Candida* species. None of the extracts showed any activity against *Candida parapisilosis* and *Candida tropicalis*.

Table 2. Anticandidal activity of methanol and water extracts of blackberry fruits and leaves against some clinic isolates.

	Mean Inhibition Zone Diameter (mm)			
	Methanol Extracts		Water Extracts	
Microorganisim	Fruit	Leaf	Fruit	Leaf
Candida albicans	8	10	_	10
Candida glabrata	_	15	_	8
Candida krusei	_	13	_	10
Candida parapisilosis	_	_	_	_
Candida tropicalis	_	_	_	_

–: Not active, inhibition zone was no greater than 6mm; (7–12 mm), moderately active; (> 12), highly active. Negative controls (Methanol and Water) showed no inhibiting effect. Inhibition zone diameters of positive controls were ranging to 12-15 mm for Amphotericin B.

The highest anticandidal activity was expressed by methanol and water extracts of blackberry leaves against *Candida glabrata*. with 15 mm inhibition zone and 0.312 mg/ml MIC value (Table 2 and 3). Only metanol extracts of fruits have anticandidall ability against *C.albicans* with 8mm inhibition zone and 2.5mg/mL MIC values but not have any activity against the other species.

Table 3. The MIC values (mg/ml) of methanol and water extracts of blackberry fruits and leaves against candida strains tested in the micro dilution assay.

	Minimal Inhibition Concentration (MIC) mg/mL			
	Methanol Extracts		Water Extracts	
Microorganisim	Fruit	Leaf	Fruit	Leaf
Candida albicans	2.5	1.25	_	1.25
Candida glabrata	_	0.312	_	2.5
Candida krusei	_	0.625	_	1.25

MIC values of positive control was ranging to 0.5-1µg/mL for 30 mg amphotericin B.

Blackberries are a rich source of natural antioxidants as they contain high levels of phenols, flavonols and anthocyanins and are therefore well-reputed scavengers and inhibitors of free radicals [18]. Halvorsen *et al.*, investigated the total antioxidant capacity of cultivated *R. fruticosus* collected at three different locations. They detected very high amount of total antioxidant concentration (5.03 to 9.17 mmol/100 g)[19]. Also, Huang *et al.*, reported that blackberry extracts exhibited a strong DPPH scavenging activity (95.37%) at 2 mg/mL [20]. It has been previously reported that phenolic compounds are called high-level antioxidants because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals. Some authors reported high amount of anthocyanins, phenolics and antioxidant activities of fresh fruit of blackberry in their studies [21-23].Therefore, our results are compatible with this previous studies.

Phenolic compounds affected the growth of microorganisims by different mechanisms, yet not well understood. It is assumed that phenolic compounds are responsible for the antimicrobial activity. The fruits of the genus *Rubus* are rich in ellagitannins, which can permeate the outer cell membrane of Gram-negative bacteria [24]. So, antimicrobial activity of berries is likely to be caused by multiple mechanisms and synergies because they contain various compounds, for example, weak organic acids, phenolic acids, and tannins and their mixtures of different chemical forms [25].

In the majority of the previously performed antimicrobial studies, whole blackberry species or some parts of plant were tested, while anticandidal activity of berries less investigated. Riaz *et al*, screened the methanol extracts for their antifungal activity against nine pathogenic fungal strains (*Yersinia aldovae*, *Aspergillus parasiticus*, *Candida albicans*, *Aspergillus niger*, *Aspergillus effusus*, *Macrophomina phaseolina*, *Fusarium solani*, *Trichophyton rubrum*, *Saccharomyces cerevisiae*) without recording any biological activity [12]. In the oposit of his study, we detected high anticandidal activity, especially methanol extracts of berry leaves against some clinic isolates of *Candida* species.

In conclusion, the current study revealed significant antioxidant activity of the extracts of both blackberry fuits and leaves. Additionaly, high anticandidal activity was recorded, especially in blackberry leaves. In addition, these results indicate that blackberry could not only serve as a rich source of food but may also be of importance in ethno-botanical studies due totheir high antioxidant and antimicrobial activities.

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