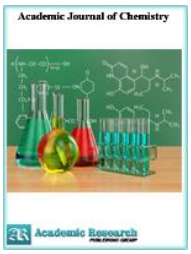

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In Vitro Elucidation Free Radical Scavenging Activity of Flavonoids Extracted From Leaves and Flowers of *Arbutus Pavarii (Ericaceae)*

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Abstract: Increasing attention has been paid to the use of natural antioxidants, particularly of plant origin in order to substitute synthetic antioxidant due to their negative health effects. The aim of this study was to screen the flavonoid extracted from leaves and flowers of *A. pavarii* and their mixture of to show any antioxidant activity *in vitro*. the antioxidant and free radical scavenging activity were determined by several standard methods using spectrophotomer. Flavonoid extracts showed higher reducing ability in comparison with ascorbic and BHA. It was found that flavonoids extracted from leaves have the most activity to reduce the DPPH with an IC_{50} of 0.625mg/mL; more effective than others. This extract also exhibited fairly good antioxidant activity by inhibiting the generation of OH (IC_{50}= 0.625mg/mL) and NO radicals (IC_{50}= 1.25mg/ml). The inhibition of lipid peroxidation was recorded in flavonoids extracted from leaves (60%). Synergism was the main effect among the combined extract, C3 (L:F; 2: 1). Three types of interactions (synergistic, additive and negative synergistic effect) were observed.

Keywords: *A. pavarii*; Flavonoids; Antioxidants; Free radicals.

1. Introduction

There is growing intendancy towards finding naturally occurring antioxidants particularly of plant origin in order to substitute synthetic antioxidant due to their negative health effects [1]. The antioxidant activity of plants are mainly due to the presence of phenolic compounds and flavonoids [2].

Flavonoids comprise the most common group of plant polyphenols, that play important roles in various metabolic processes in photosynthesizing cells, and their existence is therefore widespread in the plant kingdom [3]. Flavonoids consist six major subclasses including flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones [4]. Differences in molecular structure have been commonly related to specific biological properties of compounds that comprise the flavonoid group [5]. The flavonoids have long been recognized to possess neuro-protective, anti-inflammatory effects, anticancer, anti-genotoxic and antiglycative activities, which are basically related to their antioxidant properties [6]. The free radical-scavenging and antioxidant activity of flavonoids extracted from plants has been reviewed by Middleton, *et al.* [7]. It has been confirmed that pharmacological effect of flavonoids is correlating with their antioxidant activities [8].

Free radicals are dangerous substances produced in the body along with toxins and wastes which are formed during the normal metabolic process of the body. It has been reported that free radicals, the partially reduced metabolites of oxygen and nitrogen, are highly toxic and reactive causing tissue injury [9]. The most common reactive oxygen species (ROS) are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$) and highly reactive hydroxyl radical ($^{\bullet}OH$) [10]. Oxidation process is one of the most vital routes for producing free radicals in food, drugs and living systems [11]. Flavonoid compounds are capable to scavenge and quench various radicals (oxygen centered; carbon-centered; alkoxyl, peroxyl, or phenoxyl radicals) and ROS. They are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the ROS by reacting with the reactive compound of the radical and therefore can no longer react with biomolecules, resulting in less

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damage [12]. Hence, diseases associated with free radicals can be avoided by antioxidant therapy which gained an immense importance.

The objective of this investigation was to determine the antioxidant activity of flavonoids extracted from leaves and flowers of *Arbutus pavarii* and assess their content of flavonol, anthocyanidin and proanthocyanidin. Since folk medicine in country like Libya usually using mixture of many plants with antioxidant activity as well as many of health promoting drugs was made of more than one component, It is worthy to investigate the synergistic effect especially there is a possibility of interacting and even neutralizing the effects which is necessary to consider. In addition, the correlation between antioxidant activity and flavonol, anthocyanidin and proanthocyanidin contents "were also determined".

2. Materials and Methods

2.1. Chemicals

Chemicals including 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), Butylated hydroxyl anisole (BHA), Linoleic acid, Hydrogen peroxide, and Catechin were obtained from Sigma Chemical Company Ltd. (USA). Sodium phosphate (monobasic and dibasic), Sodium nitrite, Sodium acetate, Sodium hydroxide, Sodium nitroprusside, Potassium dihydrogen phosphate, Potassium ferric cyanide [$K_3Fe(CN)_6$], Ferrous chloride, Ferric chloride, Aluminium chloride, Ammonium molybdate, Ammonium thiocyanate, Napthylethylenediamine dihydrochloride (NED), Ethylenediaminetetra acetic acid (EDTA), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Hydrochloric acid, Glacial acetic acid, Sulfanilic acid, Sulphuric acid, Ethanol, Methanol, Ethyl acetate, Chloroform, Ascorbic acid, Rutin, 2-Deoxy-D-ribose, Vanillin, Vitamin E (α-tocopherol), and all other chemicals were of acceptable laboratory grade.

2.2. Plant Material

Arbutus pavarii (Ericaceae) (flowers and leaves) were collected in January 2015 from Al Marj, Libya. The plant was authenticated by the Botany Department, Faculty of Science, University of Tripoli, Tripoli, Libya. Fresh sample was allowed to dry at room temperature, ground into a powder, passed through a suitable mesh sieve and the dried powder was then stored at room temperature (25°C±2) until analysis.

2.3. Microwave Assisted Extraction (MAE) of Flavonoid

Experiments were carried out in a domestic (Black & Decker, Model No. MZ3000 PG, SL13YD, England) microwave oven system. Twenty five grams of the powdered plant materials were mixed with solvents (ethanol or methanol 80%) at a suitable ratio (500 mL). An intermittent microwave irradiation method was used to keep the temperature of the extraction mixtures below 80 °C [13]. The suspension was irradiated in microwave oven at regular intervals (30 sec radiation and 30 sec off). Variation in irradiation time from one plant to another was dependent on the result of an assay-guided purification i.e. a quick flavonoids TLC spot test on the extraction products, using mobile phase (7:3; v/v) benzene : methanol. Extraction was stopped when the spot test indicated maximum yield for the tested samples (data not shown). The infusions were allowed to cool down to room temperature, filtrated and stored at (4 °C) for further analysis.

2.4.1. Anthocyanidin Content (AC)

It was determined using the vanillin-HCl colorimetric method [14]. An aliquot of 0.5 mL sample was mixed with 3 mL methanol containing 0.03 g/mL vanillin in a test tube wrapped in tinfoil. Then 1.5 mL hydrochloric acid was added to the reaction solution and mixed thoroughly at room temperature. The mixture was kept in the dark place for 15 hr. The absorbance was read at 500 nm with ethanol solution containing 0.02 g/mL hydrochloric acid as a blank. The standard curve of catechin was obtained under the same conditions. Total Anthocyanidin content was expressed as mg catechin equivalent (CE) per gram of extract.

2.4.2. Proanthocyanidin Content (PC)

Total proanthocyanidins content was determined according to Li, *et al.* [15] method. A 0.5 mL of the flavonoid extracts (20 mg/mL) was added to 3 mL of (4%; w/v) vanillin in methanol and 1.5 mL of HCl (35%) and then incubated at RT for 15 min in dark place. After which, absorbance was recorded at 500 nm. Total Proanthocyanidin content was expressed as mg catechin equivalent (CE) per gram of extract.

2.4.3. Flavonol Content (FC)

Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran [16]. Two milliliters of sample, 2.0 mL of $AlCl_3$ were mixed with 3.0 mL of sodium acetate solution (50 g/L) and thereafter incubated for 2.5 hr at 20 °C. The absorption was read at 440 nm using UV/Vis Spectrophotometer.
Total flavonol content was expressed as mg rutin equivalent (RE) per gram of extract.

2.5 Estimation of Antioxidant activity

2.5.1. Reducing Power

The antioxidant activity was investigated using the ferric reducing antioxidant power (FRAP) assay for flavonoids extracted from leaves and flowers of *Arbutus pavarii* was investigated according to the method of Oyaizu [17]. Various concentrations of Flavonoid extracts, ascorbic acid and BHA (2.5 ,5, 10, 20 mg/mL) were mixed with 2.5 mL of phosphate buffer (2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (10 g/L) and then the mixture was incubated at 50° C for 20 min. After which, 1.5 mL of trichloroacetic acid (100 g/L) was added to the reaction mixture and then centrifuged at 3000 rpm for 10 min. Finally, 0.5 mL of the supernatant solution was mixed with 1.0 mL of distilled water, 0.5 mL of $FeCI_3$ (0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extracts. Phosphate buffer (PH 6.6) was used as blank solution.

2.5.2. Total Antioxidant Capacity (TAC)

The total antioxidant capacity was evaluated by the phosphomolybdenum method according to the procedure described by Prieto, *et al.* [18]. A 0.3 mL of flavonoid extracts was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95 °C for 90 min. After that, the absorbance of the green phosphate/Mo complex was measured at 695 nm. The higher absorbance value indicated higher antioxidant activity. The results were expressed as ascorbic acid equivalent using the following linear equation: [$Y = 3.9981X + 0.4436$; $R^2 = 0.9945$] where Y is the absorbance at 695 nm and X is the concentration as ascorbic acid equivalent (mg/mL). The values are presented as the means of triplicates.

2.5.3. DPPH free Radical-Scavenging Activity

The free radical scavenging activity of flavonoids extracts was measured by DPPH according to Wong, *et al.* [19] method. The initial absorbance of DPPH in methanol was measured at 515 nm until the absorbance remains constant. Briefly, 40 µL of flavonoids extracted from leaves and flowers of tested plant at different concentrations (2.5, 5, 10 and 20 mg/mL) was added to 3.0 mL of DPPH (0.1 mM) in methanol solution, shaken vigorously and allowed to stand for 30 min at room temperature. The absorbance was measured at 517 nm against the methanol blank. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging. The percentage of $^{\bullet}$DPPH scavenging activity calculated from the following equation:

% DPPH radical scavenging activity = $([A_C - A_S]/A_C) \times 100$

Where A_C was the absorbance of the control reaction and As was the absorbance in the presence of the flavonoid extracts. The results were compared with ascorbic acids and BHA as standers.

The IC_{50} was calculated as the amount of antioxidants present in the sample necessary to reduce the initial $^{\bullet}$DPPH concentration by 50%.

2.5.5. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was measured by the ability of the different fractions of extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) [20]. The reaction mixture contained; 500 µL of deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 µL of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 µL of H_2O_2 (200 mM) with or without the extract solution (100 µL). The reaction was triggered by adding 100 µL of 300 mM ascorbate and incubated for 1.0 hr at 37°C. 0.5 ml of the reaction mixture was added to 1.0 mL of TCA (2.8%; w/v), then 1.0 mL of 1% TBA were added to the reaction mixture. The mixture was incubated for 15 min on a boiling water bath. After cooling, the absorbance was noted at 532 nm against a blank (the same solution but without reagent). The scavenging activity on hydroxyl radical was calculated as follows:

$$\% \text{ Hydroxyl radical scavenging activity} = ([A_C - A_S]/A_C) \times 100$$

2.5.4. Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity was measured spectrophotometrically according to Garrat [21] method. A 1.0 mL of Sodium nitroprusside (10 mM) in phosphate buffer was added to 0.5 mL of sample and incubated at 25 ºC for 150 min. Thereafter, 0.5 mL of the reaction mixture containing nitrite ions was removed and added 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid), shaked and allowed to stand for 5 min. then 1.0 mL of NED (0.1%) was added, mixed and allowed to stand for 30 min. The absorbance of the mixture was measured at 540 nm against the corresponding blank. Ascorbic acid and BHA were used as positive control. The percentage of nitric oxide scavenging activity was calculated by the following formula:

$$\% \text{ nitric oxide radical scavenging activity} = ([A_C - A_S]/A_C) \times 100$$

2.5.6. Effect of Flavonoid Extracts on the Peroxidation of Linoleic Acid

The antioxidant activity of the extracts against lipid peroxidation was determined using FTC and TBA methods. The FTC method was used to evaluate the peroxides at the initiation of lipid peroxidation, and TBA method was used to evaluate the secondary products of peroxide oxidation such as aldehyde and ketone.

2.5.6.1. Ferric Thiocyanate (FTC)

The antioxidant activity of the flavonoid extracts from leaves and flowers of *Arbutus pavarii* was determined according to the ferric thiocyanate method in linoleic acid emulsion [22]. A mixture containing 4.0 mg of the sample [or methanol (as control) or BHA/vitamin E (as standard)] in 4 mL of pure ethanol (99.5%), 4.1 mL of linoleic acid (2.52%) in pure ethanol, 8 mL of phosphate buffer (0.05 M, pH 7.0) and 3.9 mL of distilled water was placed in a vial with screw cap and then placed in a rotary incubator (150 r/min, 40º C) in a dark place. To 0.1 mL of this mixture 9.7 mL of ethanol (75%) and 0.1 mL of ammonium thiocyanate (30%) were added. Precisely 3 min later the addition of 0.1 mL of ferrous chloride solution (20 mM in 3.5% HCl) acid was added to reaction mixture; (the absorbance of red color indicated the antioxidant activity) was measured at 500 nm for every 24 hr until the absorbance of the control reached maximum. The percent inhibition of linoleic acid peroxidation in an emulsion was calculated following the equation:

$$\% \text{ inhibition of peroxidation (\% IP)} = ([A_C – A_S]/A_C) \times 100$$

2.5.6.2. Thiobarbituric Acid Reactive Substances (TBARS)

TBA method was performed as described by Ottolenghi [23]. Briefly, 2.0 mL of trichloroacetic acid (20%) and 2.0 mL of tiobarbituric acid (0.67%) were added to 2.0 mL of the mixtures containing the sample prepared in the FTC method. This mixture was kept in water bath (100 ºC) for 10 min and after cooling to room temperature, was centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant, containing TBA-MDA complex was read at 532 nm. The anti-lipid peroxidation activity (%) was calculated using the formula :

$$\% \text{ Anti-lipid peroxidation activity} = ([A_C – A_S]/AC) \times 100$$

2.6. Statistical Analysis

Data were expressed as means ± standard deviations (SD) of triplicate determinations. All statistical analyses were carried out using SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). Statistical differences between extract activities were determined using ANOVA followed by Least Significant Difference (LSD) testing. Differences were considered statistically significant when $P<0.05$. The Pearsons correlation analysis was performed between antioxidant activity, total flavonols, anthocyanidin and proanthocyanidin contents.

3. Results and Discussion

Recently, there is great interest in using natural antioxidants in industrial and health fields. Among different secondary metabolites of the plants, flavonoids are the main compounds with considerable antioxidant activity. They are the main components of many products and are sometimes used in combinations [24, 25]. Since, synergistic effect of combination of compounds often exceed their individual performance , evaluating the effect of their combination is important.

Antioxidant properties of flavonoids have been attributed to various reactions and mechanisms such as prevention of chain initiation of lipid peroxidation, binding of transition metal ion catalysts, reductive capacity, and or radical scavenging effect [26, 27]. Therefore, in this study various methods have been used to determine the antioxidant activity of flavonoids extracted from leaves and flowers of *A. pavarii* and their combination in order to allow rapid screening of extracts as substances that have low antioxidant activity *in vitro*, may likely show little activity *in vivo* [2].

3.1. Reductive Activity

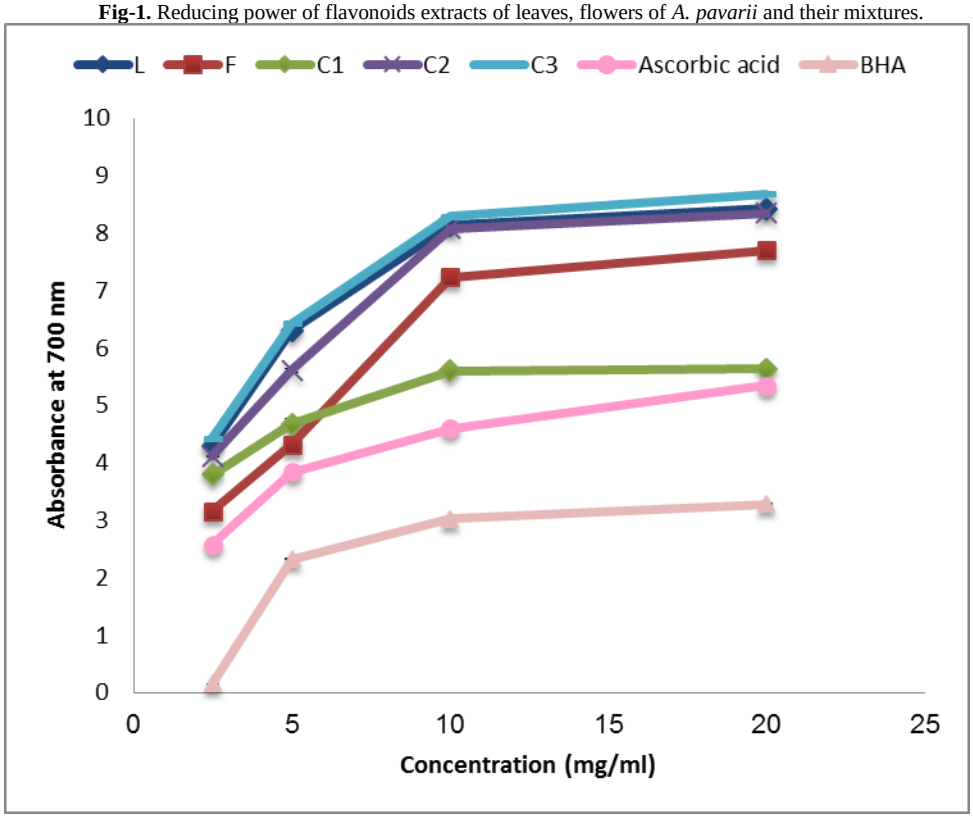
3.1.1. Reducing Power Activity

The reducing capacity of flavonoids extracts may serve as a significant indicator of its potential antioxidant activity [28]. The presence of reducing agents in the test samples would result in reducing Fe^{3+} ions of ferricyanide complex to the ferrous form (Fe^{2+}). In this study reducing power of flavonoids extracts and their combinations was conducted and the synergistic effects of combined extracts at three different mixing ratio (1:1 (C1), 1:2(C2) and 2:1 (C3), on reducing iron was also studied (Fig 1).

The reducing power of the tested extracts increased with increase in concentration and the combination gave maximum synergism as compared to each one individually. This result was in concord to previous studies [29, 30]. Flowers and leaves extracts as well as their combined extracts C1 and C2 were more efficient on reducing iron than positive controls (ascorbic acid and BHT) (Fig 1). Flavonoid extracts of leaves and flowers showed highly reducing ability in comparison with their crud extracts [31]. Flavonoids are well known reductant substances *via* donating a

hydrogen atom to break the free radical chain as well as they react with certain precursors of peroxide, thus preventing peroxide formation [32, 33].

Fig-1. Reducing power of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures.

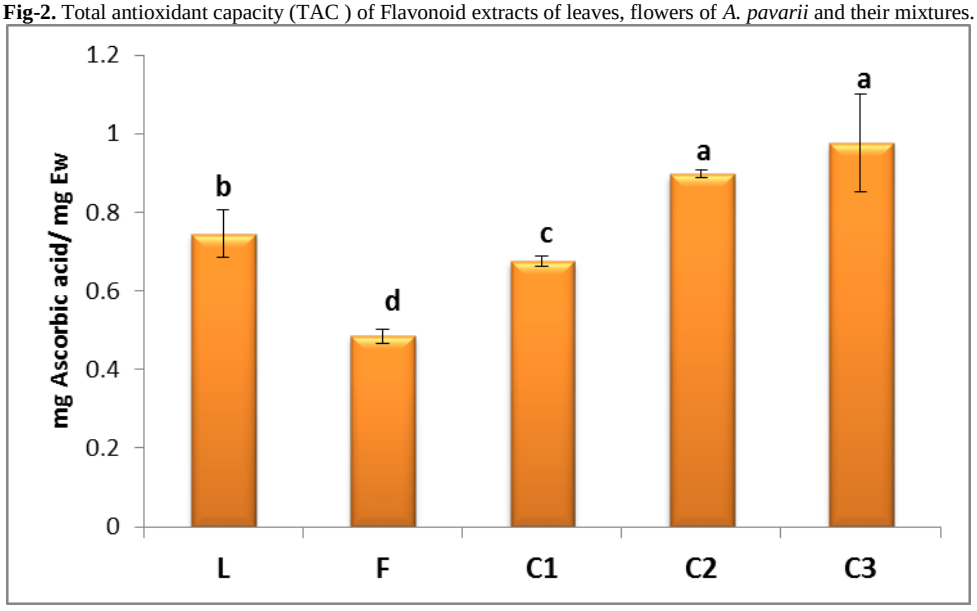


Each value is represented as mean ±SD (n=3). L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2) BHA:

3.1.2. Total Antioxidant Activity

The reductive properties of flavonoid extracts was also studied using total antioxidant capacity which is based on the reduction of Mo (VI) to Mo (V) (Figure 2). The total antioxidant capacity (TAOC) results were similar to reducing power results; the maximum antioxidant activity was obtained from C3 sample (0.976 mg of Ascorbic acid/mg extract weight) and the lowest activity was recorded in flower extract (0.484 mg of Ascorbic acid/mg extract weight) (Fig 2). the antioxidant activity of combined extract (C3) was significantly higher than individual extracts (P<0.05). Previous studies have shown that many polyphenols including flavonoids contribute significantly to the phosphomolybdate scavenging character of medicinal plants [34].

Fig-2. Total antioxidant capacity (TAC) of Flavonoid extracts of leaves, flowers of *A. pavarii* and their mixtures.



Each value is represented as mean ±SD (n=3). Means with different letters (for each level) are significantly different at $P < 0.05$. L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2)**.** DPPH radical scavenging activities (% ± SD)

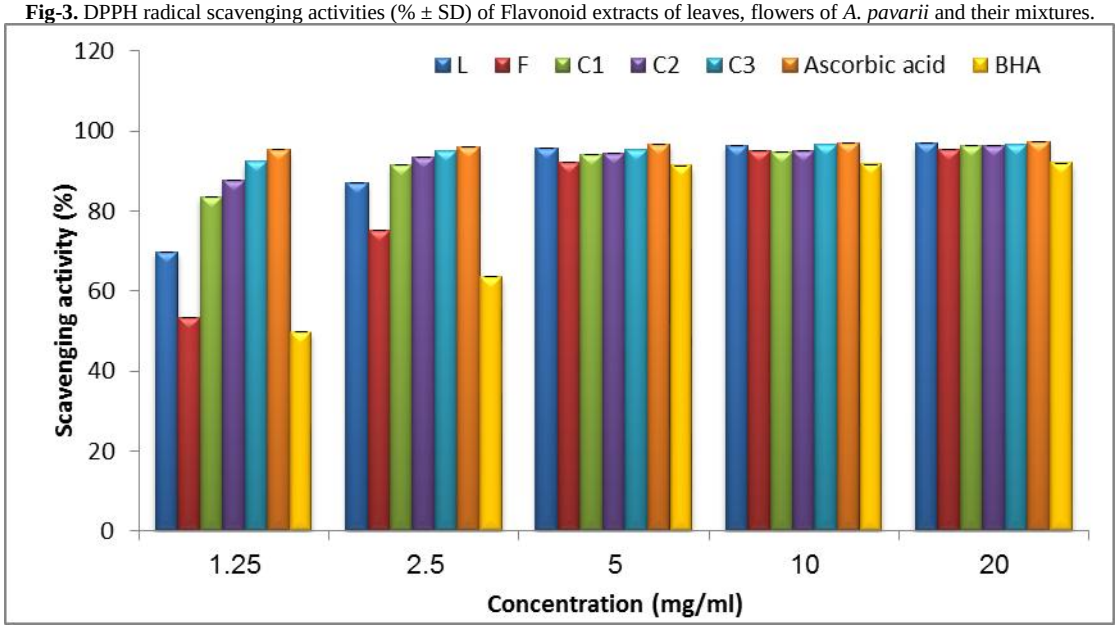
3.2. Radical Scavenging Activity

Antioxidants neutralize toxin and volatile free radicals that are defined as atoms or groups of atoms having an unpaired electron [35]. These also include related reactive oxygen species (ROS) that leads to free radical generation, causes the cascading chain reaction in biological system. In a normal healthy organism or human body, the generation of pro-oxidants in the form of ROS is effectively kept in check by various levels of antioxidant defense. Antioxidants present in various dietary supplements offered their beneficial effects by neutralizing these ROS during various disease conditions. Lipids, proteins and DNA are all susceptible to attack by free radicals and cellular damage induced by oxidative stress has been implicated in the etiology of numerous diseases.

3.2.1. DPPH Radical Scavenging Activity

DPPH radical is widely used as a model to investigate the scavenging potential of several natural compounds such as phenolic and anthocyanins or crude extracts of plants [36]. In present study, flavonoid extracts scavenged the DPPH stable free radicals in a concentration dependent manner (2.5-20 mg/ml) (Fig 3). At low concentrations(1.25 and 2.5 mg/ml), the effect of flavonoid extracts on DPPH radical was significantly higher as compared with ascorbic acid and BHA ($P<0.05$) while no significant results was observed at high concentrations (Figure 3). This can naturally be attributed to the content and more possibly to the composition of endogenous antioxidant components of extracts [37]. There was a direct relationship found between an increase in concentration and scavenging activity, up to 5 (mg ml^{-1}), but higher concentrations had no effect in increasing the activity. The free radical scavenging activity is due to hydrogen and/or electron donation [32] which might prevent reactive radical species. The ability of tested extracts and their combined extracts to reduce DPPH radicals, supports its free radical scavenging activity. The present study indicates the proton donating property may be responsible for free radical scavenging activity of investigated extracts. This activity is believed to be mainly due to their redox properties which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.

Fig-3. DPPH radical scavenging activities (% ± SD) of Flavonoid extracts of leaves, flowers of *A. pavarii* and their mixtures.

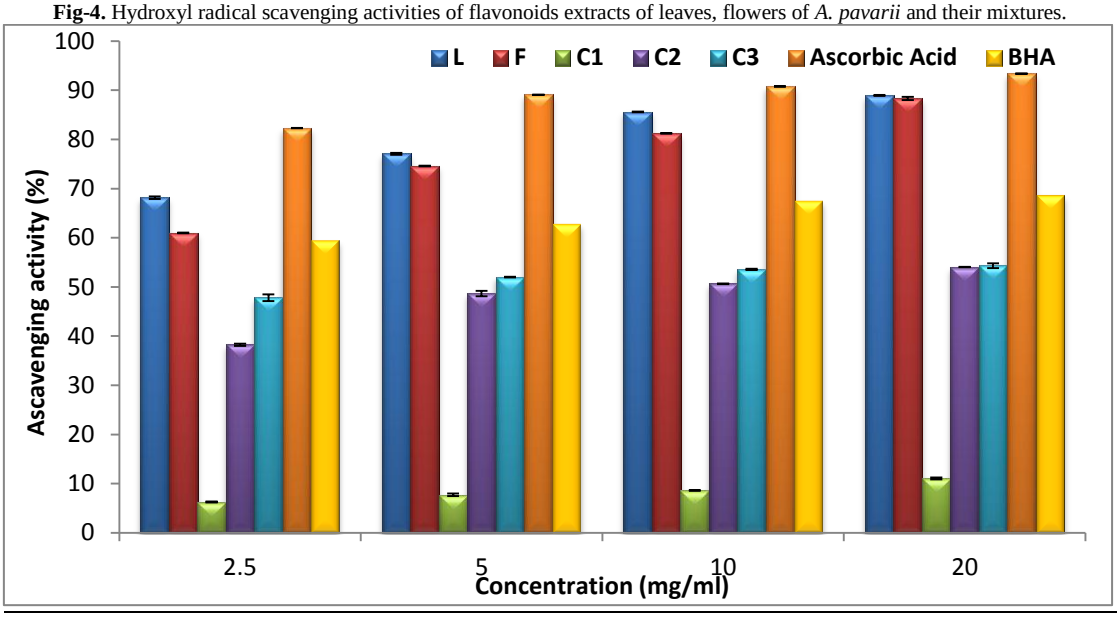


Each value is expressed as the mean ±SE (n=3). L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2)**.**

3.2.2. Hydroxyl Radical Scavenging Activity

Hydroxyl radical, one of the most harmful reactive oxygen species in living systems, reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids leading to cellular damage [34]. The hydroxyl radical scavenging activity of extracts is presented in Fig 4. The ability of tested samples to scavenge hydroxyl radical is directly proportional to its antioxidant property that is evident from the low intensity of red colour. Hydroxyl radicals were effectively scavenged and 2-deoxyribose was prevented from degradation by the extracts when added to the mixture. Flavonoids extracted from leave showed significantly higher scavenging activity as compared with other extracts ($P<0.05$) but it was less activity as compared with ascorbic acid (Fig 4).

Fig-4. Hydroxyl radical scavenging activities of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures.



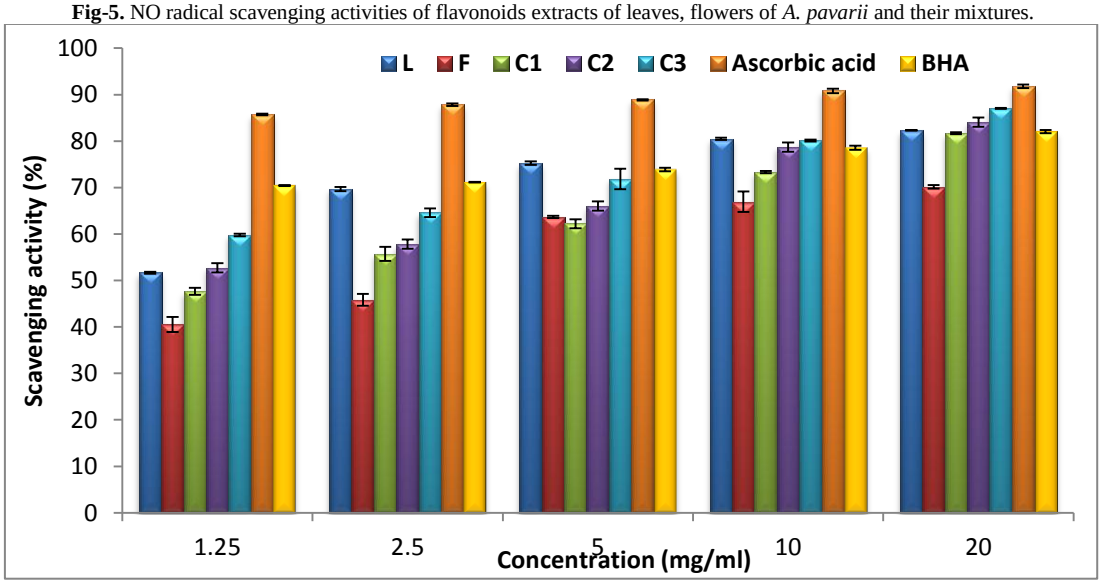
Values are means of triplicate determinations ± standard deviation. leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2).

3.2.3. NO Radical Scavenging Activity

NO is generated in biological tissues by specific nitric oxide synthases (NOS), which metabolizes arginine to citrulline with the formation of NO• via a five electron oxidative reaction [38]. Incubation of with extracts lead to suppression of NO• release and that may be attributed to a direct NO• scavenging effect as all the flavonoids extracts decreased the amount of nitrite generated from the degradation of sodium nitroprusside *in vitro*. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. Many flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity [39].

Among the extracts examined, Flavonoids extracted from leave (L) sample and combined extract sample (C3) exhibited a higher scavenging activity in comparison with other samples. However, their activity was less than ascorbic acid (Fig 5). It was also found that the concentration of compounds and the ratio at which they are mixed are important variables in defining the antioxidant capacity and synergistic mode.

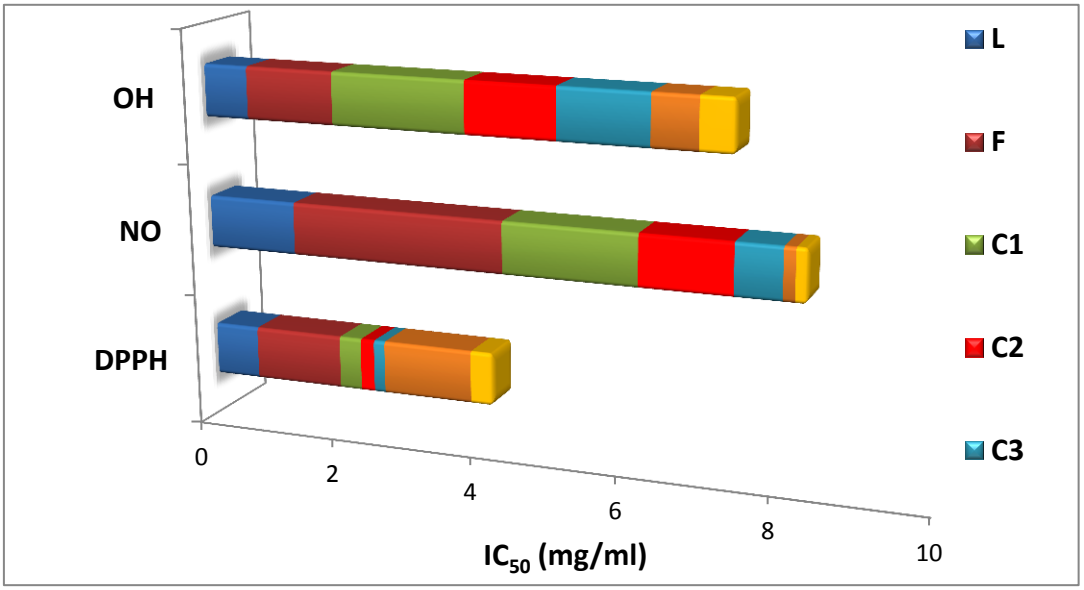
Fig-5. NO radical scavenging activities of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures.



Values are expressed as means ± SD. L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2).

The DPPH, OH and NO IC50 values (IC50 value is the concentration of the sample required to inhibit 50% of radical) of the flavonoid extracts and their mixtures were calculated (Figure 6). IC_{50} of combined extracts sample (C3) was significantly lower than others ($P<0.05$) which indicating that C3 was the most effective on free radical scavenging activity (Fig. 6).

Fig-6. IC_{50} values of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures.



L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2).

3.2.4. Antioxidant Activity Determination in Linoleic Acid System

Marinova, *et al.* [40] suggested that, as lipid oxidation is a complex and multi-stage process, antioxidant activity in preventing each one of these stages should be studied; because, hydroperoxide production and destruction are separable stages that can be affected by antioxidants in some completely differing mechanisms. Therefore, using extracts in complex nature of lipid system influences its activity and type of interaction. Also the effect of antioxidants depends on many factors such as mixing ability, and activity in different lipid systems [41].

In present study, the ferric thiocyanate (FTC) method measures the amount of primary product of lipid peroxidation (peroxides), while TBA test is used to measure the secondary products of oxidation (MDA). Results related to a measurement of inhibition of peroxidation status during a 7 days period of a day intervals are presented in Fig 7. There were significant differences observed between antioxidant ability of different extracts and controls (BHA and vitamin E) indicating the capability of these extracts to be used as natural antioxidants and substitute the synthetic antioxidants.

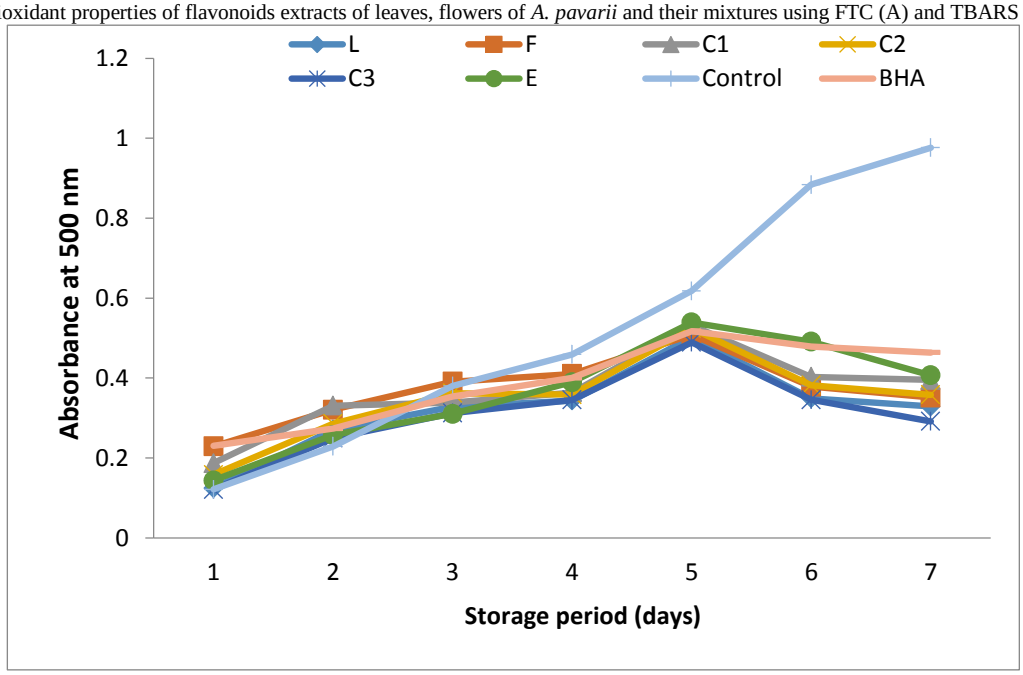
Anti-lipid activity results of tested extracts and their combination at concentration (4mg/ml) showed high inhibition was an effective antioxidant compared with control. There was a significant increase in primary oxidation adducts for negative control that untreated with extracts compared extracts treated samples (P<0.05) (Fig 7A). There was a significant increase in primary and secondary oxidation products during storage time but this trend changed after 5 days when the values dropped dramatically in all samples except in control sample (Fig 7B). A decreased in FTC values might be due to hydroperoxides degradation to secondary lipid peroxidation products [42]. During the oxidation process, peroxides are gradually decomposed to lower molecular weight compounds. One such compound is malondialdehyde, which is measured by the TBA method [43]. There was a substantial differences between samples incubated with flavonoid extracts and control samples (Vitamin E and BHA) in reducing the percentage of inhibition of lipid peroxidation products (P<0.05) (Fig 8). The combined extracts sample showed the lowest TARBS levels as compared with other samples. The current results revealed that flavonoids extracts used at the right concentration may delay or inhibit lipid oxidation and can be used as natural antioxidants in oil food system and was in agreement with previous report.

3.3. Synergistic Action of Extracts

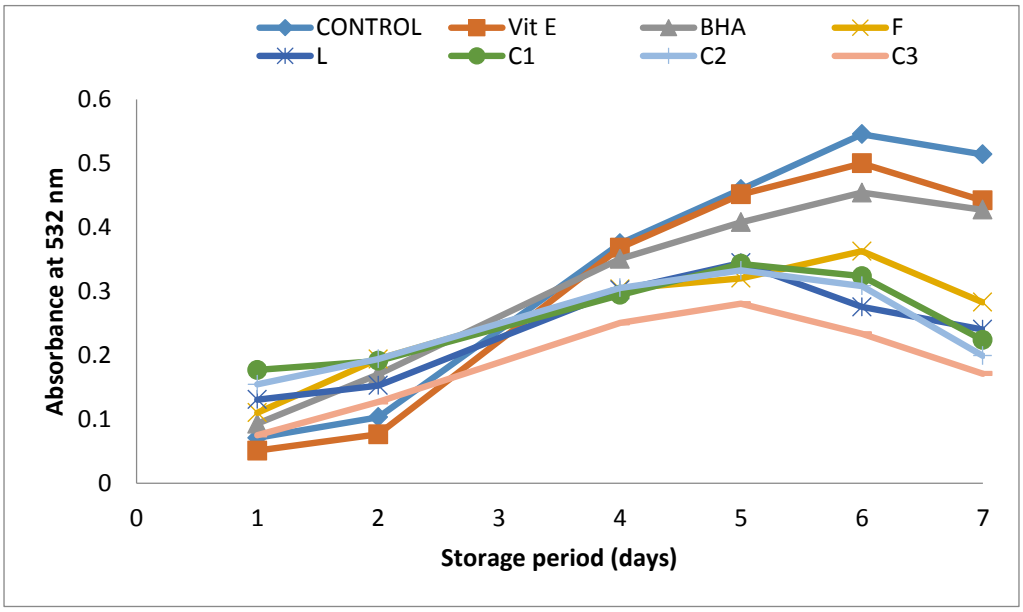
Concerning the synergistic effect of the combined extracts, the results in Table 1 showed that mixing of flavonoids extracted from leaves and flowers at ratio 2:1, L: F appreciably enhanced the antioxidant activity. This indicates that leaves flavonoids extract when used in combination with flowers extract improved the radical scavenging effect as compared with their individual effect.

In order to investigate if synergistic interactions occurred when leaves flavonoids extract and flower flavonoids extract were mixed with different (F:L; 1:1, 1:2 and 2:1), theoretical versus experimental values of antioxidant activity IC_{50} (mg/mL) of the combined extracts were assessed. Based on the data obtained from the individual antioxidants, the theoretical values (TAC) were calculated. If the experimental values (EAC) is the same as the theoretical value, then the contribution of the individual antioxidant would be additive. If the experimental value is greater than the theoretical value, then an interaction happened among the antioxidants, thus displaying synergism. simply, when a ratio of EAC/ TAC >1, it would indicate an synergism effect (SE). The SE of the combined extracts, C1, C2 and C3 presented in Table 2.

Fig-7. Antioxidant properties of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures using FTC (A) and TBARS (B) assays.



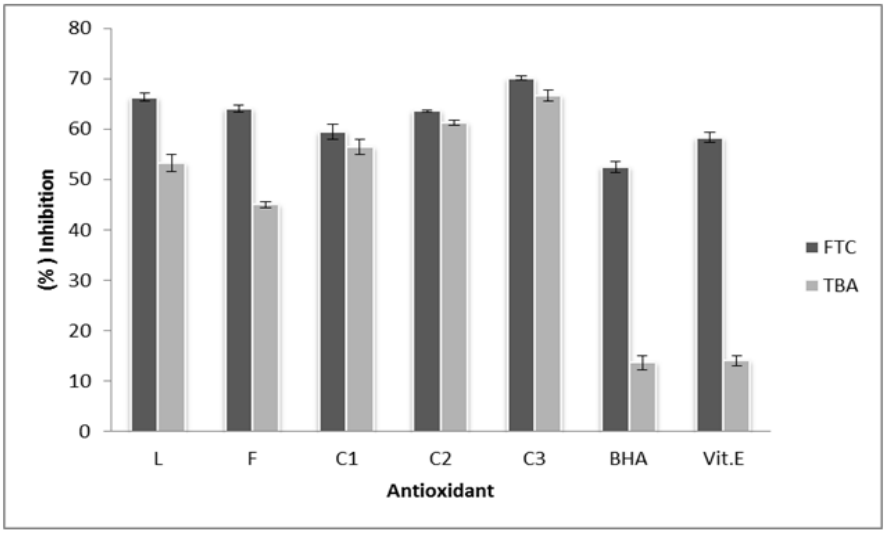
(A)



(B)

Values are expressed as means ± SD**.** L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2)**.**

Fig-8. Inhibition (%) of Lipid peroxidation by flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures at 4mg/mL.



Values are expressed as means ± SD**, n=3.** L: leaves, F: flowers; C1: combined extracts (L:F, 1:1); C2: combined extracts (L:F, 2:1); C3: combined extracts (L:F, 1:2)**.**

The synergistic effects of DPPH radical scavenging capacity were greater than 1 for all the combinations. However, significant synergistic effect ($P<0.05$) was produced only by the mixtures C2 and C3, in which the concentration of induced a synergistic effect was 5.0 and 2.5 mg/mL, while the highest synergistic effects of NO• scavenging activity was recorded for C3. Using the antioxidant assay it was found that the synergism was significantly affected by dose ratios [44].

In several ternary combinations, antagonistic effect was observed and antioxidant activity had decreased. The combination of leaves and flowers with all ratios (C1, C2 and C3) showed the most decrease in •OH scavenging activity value but no synergism effect was observed. On the other hand, after being combined, leaves and flowers extracts at different ratios showed additive affect in controlling lipid peroxidation (Table 1).

Many reports have been studied the synergistic effects of plant/plant mixtures on antioxidant activity [45-48]. The synergistic effect, might be due to electron transferring from the component of low antioxidant activity to the component with a higher antioxidant activity and a retrieval of this component so that it can give its hydrogen group to another free radical and continue the process. Changing mixing ratio between two components could influence these interactions leading to either decrease or increase the antioxidant activity [48]. Such interactions resulting from mixing different flavonoids have been evaluated by Hidalgo, *et al.* [49]. Also studies on oxidative behavior of plant mixtures in a linoleic acid model system and a palm olein system were showed a synergistic effect [46].

Table-1. Experimental and theoretical scavenging capacity values of flavonoids extracts of leaves, flowers of *A. pavarii* and their mixtures at their different combinations.

	•DPPH			**NO•**		
Mixtures	**aTheoretical**	**Experimental**	**Effect**	**Theoretical**	**Experimental**	**Effect**
C1	0.47	0.31a	S	2.5	1.875	A
C2	0.47	0.20b	S	2.5	1.25	S
C3	0.47	0.16b	S	2.5	0.625	S
	•OH			**Lipid peroxidation**		
Mixtures	**Theoretical**	**Experimental**	**Effect**	**Theoretical**	**Experimental**	**Effect**
C1	0.94	1.87	NS	4.10	3.54	A
C2	0.94	1.25	NS	4.10	3.26	A
C3	0.94	1.25	NS	4.10	2.99	A

[a] The theoretical values were obtained considering additive contributions of the individual species.
A: additive effect; S: synergistic effect; NS: negative synergistic(antagonist) effect

3.4. Quantitative Analysis of Flavonoid extracts

The flavonoids form a group of approximately nine thousand plant metabolites. Chemically, they can be classified as polyphenols or phenolics. Subgroups of flavonoids include flavones, flavonols, flavanones, chalcones, aurones, isoflavones, anthocyanins, and proanthocyanidins. These compounds play many physiological roles in plants and some of them are also favorable to human health due to their high redox potential [6]. In this work, flavonoid extracts were subjected to quantitative analysis of flavonol, anthocyanidin and proanthocyanidin and were also evaluated for an increase in their contents with an increase in antioxidant activity.

The flavonol (TC), anthocyanidin (AC) and proanthocyanidin (PC) contents in leaves extract were significant higher as compared to flowers (Table 2). Proanthocyanidin content was markedly higher in leaves in comparison

with its content in flowers extract. Higher content of proanthocyanidin in leaves resulted in noticeably reduction in ROS and nitrite levels. In previous study, proanthocyanidin was exhibited scavenging abilities and even superior to that of the vitamins C and E [50].

Table-2. Flavonol, Anthocyanidin and Proanthocyanidin contents of flavonoids extracted from leaves and flowers contents.

Samples	Total Flavonol content (FC) *	Total Anthocyanidin content (AC) **	Total Proanthocyanidin content (PC) **
Leaves	$187.97^{a} \pm 0.786$	$906.85^{a} \pm 0.426$	$1250.02^{a} \pm 0.756$
Flowers	$59.24^{b} \pm 0.437$	$127.65^{b} \pm 0.569$	$364.06^{b} \pm 0.786$

* mg rutin equivalents g−1 DW;
** mg catechin equivalents g−1 DW;
*** Each value represents mean of three replicates;
SEM: standard error of the mean; Means in the same column with different superscripts are significantly different ($P < 0.05$).

3.5. Correlation between IC_{50} values of antioxidant activities and flavonoids contents

Furthermore, quantitative analysis was also carried out for investigating the correlation between antioxidant activities (IC_{50}) and flavonoids contents (flavonol, anthocyanidin and proanthocyanidin)in different extracts of *A. pavarii* (Table 3).

Pearson's correlation coefficient was positively high if 0.61 □□r □□0.97 and negatively high if -0.61 □□r □□-0.97 [53].For DPPH assay, Anthocyanidin content in both flowers(F) and combined extracts (C3) samples showed a significant positive correlation (r=0.998, 0.842* respectively, $P<0.01$) (Table 3).

The current research findings were in agreement with the results of Radovanović, *et al.* [51] which reported the strong correlation between total anthocyanins and DPPH˙ scavenging ability. While for NO assay, the highly significant correlation was observed with PC in C1 and C3 samples (r=-0.999*, 0.949* respectively, p<0.01) (Table 3). On the other hand, for FTC and FC in (C2) sample showed high significant negative correlation (r = -0.999*). Meanwhile, TF showed significant negative correlation (r = -0.999*) in combined extracts (C2). Also significant correlations of AC, and PC in the combined extracts (C3) were observed (r = - 0.998*, -1.000**, $P<0.01$, $P<0.001$ respectively) (Table 3).The highest significant negative correlation between AC and IC_{50} of •OH (r=-0.997*, p<0.01) was given by combined extracts (C1) sample while the highest positive correlation between PC and IC_{50} of •OH was given by combined extracts (C3) sample (r=0.949*, $P<0.01$). While The highest and negative between PC and IC_{50} of TBA scavenging capacity (r = -0.998*, $P<0.01$) was given by flavonoids extracted from flowers of *A. pavarii* (Table 3). It was clearly that high content of flavonols, anthocyanidins and proanthocyanidins in flavonoids extracted from extracts contributed to enhancing the antioxidant properties.

4. Conclusion

The flavonoids extracted from leaves and flowers of *A. pavarii* and their combination showed varied levels of antioxidant activity in all the *in vitro* models of antioxidant assays studied. The results from various free radical scavenging systems revealed that flavonoids extracted from leaves had significant antioxidant activity and free radical scavenging activity. To some extent flavonol, anthocyanidin and proanthocyanidin compounds present in extracts showed good correlation with antioxidant activity. Since *in vitro* antioxidant assays are based on *in vitro* chemical reactions where it is different to actual physiological systems, further evaluation of their antioxidant activities in living models is required.

Table-3. The Pearson correlation coefficients (r) between antioxidant activities (IC_{50}) and flavonoids contents.

	Flavonoids extract (L)					**Flavonoids extract (F)**				
	DPPH	**NO**	**OH**	**FTC**	**TBA**	**DPPH**	**NO**	**OH**	**FTC**	**TBA**
FC	-0.746	0.827	0.468	.0470	-0.847	0.529	0.169	-0.980	-0.564	0.985
AC	-0.140	-0.926	0.476	0.805	0.027	0.998*	-0.708	-0.728	-1.000**	0.710
PC	0.867	0.274	0.476	-0.963	.7710	-0.619	-0.060	0.996	0.651	-0.998*
	Combined flavonoids extract (C1)					**Combined flavonoids extract (C2)**				
	DPPH	**NO**	**OH**	**FTC**	**TBA**	**DPPH**	**NO**	**OH**	**FTC**	**TBA**
FC	-0.701	.1030	.998*0	.9840	-0.965	-0.906	0.455	-0.705	-0.999*	0.319
AC	0.707	-0.111	-0.997*	-0.982	.9670	.3490	.3000	.0050	.7410	-0.892
PC	.7610	-0.999*	-0.008	.1100	.3320	0.572	-0.955	0.819	.1310	0.880
	Combined flavonoids extract (C3)									
	DPPH	**NO**	**OH**	**FTC**	**TBA**					
FC	0.703	-0.547	-0.289	0.269	0.737					
AC	0.842*	-0.933	-0.997	0.998*	-0.507					
PC	-0.867	*0.949	*0.999	-1.000**	0.465					

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed)
FC: flavonol, AC: anthocyanidin and PC: proanthocyanidin

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