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IMPACT OF GROWTH STAGES ON PHYTOCONSTITUENTS, HPLC ANALYSIS AND INVITRO BIOLOGICAL ACTIVITY OF *RUMEX VESICARIUS* GROWN IN RAFHA, SAUDI ARABIA

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ABSTRACT

Rumex vesicarius belongs to the family Polygonaceae and is a traditional herb used for the treatment of various diseases and disorders. The current research was focused on evaluating the influence of the stage of plant growth on phytochemistry and biological activity. The hydroalcoholic extracts of frontal and mature leaves were evaluated for total phenolic acid by the Folin ciocalteu method and flavonoid content by the Aluminum chloride method. The physicochemical parameters like ash values, extractive value, amount of moisture, loss on drying, and crude fibers were evaluated using standard methods. The antioxidant activity was estimated by 1, 1-Diphenyl-2-picrylhydrazyl and Hydrogen Peroxide Scavenging method. HPLC study was carried out using gallic acid and quercitin as biomarkers. Total phenolic acid and total flavonoid content were more in mature leaf i.e., 290.66±0.85 µg/ml and 16.33 ±0.70. HPLC investigation exhibited more area under the curve for quercitin and gallic acid in the mature leaf. The extract of the mature leaf exhibited good antioxidant activity when compared to the frontal leaf extract. The variance in the activity was credited to the stages of development which play a key role in the production of plant metabolites. This could aid in ascertaining the best growth stage for harvesting the plant.

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Introduction

Secondary metabolites may vary in quality and quantity depending on several factors like period of harvesting, developmental stage, rainfall, type of soil, post-harvest methods, etc [1]. Many of the metabolites are only formed in particular growth stages, while in some cases their contents may increase or sometimes decrease during the various stages of plant growth. Furthermore, various researchers have reported that medicinal plants grown in various environments and stages produce disparity in plant metabolites which have an influence on their biological activities [2]. Secondary metabolites like phenolic acids, glycosides, alkaloids, flavonoids, and tannins are some of the major constituents present in biologically active herbs [3]. Hence quantification of some of these constituents will help in understanding the influence of plant stages on the phytoconstituents as these constituents are important in health care [4]. Herbal medicine is a crucial part of Arab ethnomedicine. The flora is made up of various plants of medicinal value and has been neglected due to its dry climate. The choice of plants for research depending on ethnobotanical knowledge can lead to uncovering of medicines with healing potential [5]. Lately, there is an increase in the research on phytochemistry; however, the data regarding the effect of plant stages on the phytoconstituents is not sufficiently available in the literature. The current research was planned with a focus on the impact of plant stages on the plant chemicals of the herb under consideration and its consequent effect on biological activity. This could aid in ascertaining the best stage at which this particular plant possesses the highest phytoconstituents and thereby help in the harvesting of the plant at the appropriate stage of development.

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Rumex vesicarius. belongs to the Polygonaceae family. It is named Humaidah in Arabic. The plant is erect with leathery leaves. The margins of the leaf may be entire or crenate. Flowers form clusters above the leaves [6]. The color of the flowers may vary from pink to red. It is used as medicine in the treatment of liver diseases, nausea, pain, anti-inflammatory, antitumor, anti-schistosomal, aphrodisiac, and anti-microbial activities [7-9].

Materials and Methods

Collection

The frontal and mature leaves of *Rumex vesicarius* were collected from the city of Rafha, Northern Border Province, Saudi Arabia. The plant was identified and authenticated by Dr.Heba Salem, Department of Pharmacognosy, Northern Border University, Rafha, Saudi Arabia. The leaves were rinsed with water and air-dried for around 20 days at normal room temperature. The dried material was coarsely powdered and preserved in plastic-sealed boxes to prevent any contamination.

Extraction

Ethanol and water were used in a ratio of 1:1 as a solvent for extraction. Approximately 250g of the coarse powder was extracted overnight at room temperature. The Whatman filter paper was used to filter. These extracts thus obtained were concentrated further in a rotary evaporator to get semi-solid crude extracts. Henceforth the Frontal/immature leaf extract and the mature leaf extract will be referred to as FL ex and ML ex.

Proximate Analysis [10-12]

a. Moisture Content

10 g of sample powder was placed into the crucible and subjected to drying for 3 hours. The following formula was used to estimate the moisture content.

$$\text{Percent moisture content} = \frac{Weight\ of\ sample\ -\ Weight\ of\ dried\ sample}{Weight\ of\ sample} \times 100 \quad (1)$$

b. Ash Values

Total Ash Content: The method used for the estimation of Total ash is as available in the literature. About 5 g of prepared leaves were incinerated in a crucible at 450°C in a muffle furnace until free from carbon; the ash thus obtained was placed in a desiccator cooled and weighed. Total ash was estimated with the below-given formula reference to air-dried material.

$$Percent\ ash\ content\ = \frac{Weight\ of\ ashed\ sample}{Weight\ of\ sample\ taken} \times 100 \quad (2)$$

Acid Insoluble Ash: The ash obtained was placed in a crucible and heated for around 10 minutes in 25 ml of 2N HCl. The insoluble material was collected and filtered through ash-free filter paper. This insoluble content was washed with warm water, dried, and burned up to get a constant weight, this was followed by cooling in a desiccator. Finally, it was weighed.

Water Soluble Ash: A known amount of ash was boiled with 20 ml of water. The matter which was not soluble was taken and placed on ashless filter paper, this was then washed using hot water, allowed to cool, and then weighed. The difference between the weight of the insoluble matter and the weight of the total ash gave the amount of water-soluble ash.

c. Extractive Values: The leaf material was extracted with ethanol and water (maceration). The contents of the flask were filtered, evaporated, and weighed after 24 hours. Percentage with calculated based on the amount of plant material taken.

d. Crude Fiber: Samples were treated with 150 ml of 0.2N H_2SO_4 for half an hour. The acid was drained and the sample was washed using hot water. The fiber thus obtained was dried and ignited at 550°C for 3 hours followed by cooling in the desiccator. The following equation is used to compute the percentage of crude fiber

$$\%\ \text{Crude fiber}\ = \frac{\text{Loss of weight on ignition}}{\text{Weight of the sample taken}} \times 100 \quad (3)$$

Analysis of Total Phenolic Content and Total Flavonoids

Total phenolic content and total flavonoid**s** were determined spectroscopically [13, 14].

The total phenolic content present in the ML ex and FL ex of *Rumex vesicarius* was evaluated by the Folin ciocalteu method. Standard was prepared by weighing and dissolving 20 mg of gallic acid and dissolving it in 20 ml of methanol to get a concentration of 1mg/ml. Gallic acid was prepared as aliquots of 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml, and 700 µg/ml using methanol as solvent. One ml of each of these concentrations of gallic acid and the test samples were pipetted into separate 25 ml standard flasks along with 9 ml of distilled water, followed by the addition of 1 ml of Folin ciocalteu reagent. Five minutes later, 10 ml of 7% standard sodium carbonate solution was added to the flasks

finally distill water was added up to 25 marks in the volumetric flask. APEL/PD-303UV spectrophotometer was used to record the absorbance at 750 nm using. A standard calibration curve for gallic acid was obtained and Phenolic content was estimated which was expressed as gallic acid equivalent. The measurement was carried out in triplicate.
b) Total flavonoid content in the samples was estimated by the Aluminum chloride colorimetric method. Standard quercetin and the sample were prepared using methanol as solvent. The extracts were mixed with 1.5 ml of methanol, 0.1 ml of 10 % aluminum chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water. APEL/PD-303UV spectrophotometer was used to measure absorbance at a wavelength of 415 nm. Suitable dilutions were used for obtaining a calibration curve for the standard quercitin and this was used for the estimation of the total flavonoids. The measurement was carried out in triplicate.

HPLC Analysis
Gallic acid and quercetin constituting the two important classes of phytoconstituents i.e. phenolic acids and flavonoids were used as biomarkers for HPLC analysis. The instrument used was Shimadzu Prominence and the software used was lab solutions.
For Gallic acid, the following conditions were used i.e., column: Phenomenex C18 (250x4.6,5µm), flow rate 1ml/mt, detector 272nm, injection volume 20µl column temperature 35°C. Mobile phase 50ml of acetonitrile with 200ml of water and pH was adjusted to 3 with dilute orthophosphoric acid.

Standard and Sample Preparation: 5 mg of standard gallic acid was weighed and dissolved in water 9:1 methanol, followed by sonification and filtering using 0.45-micron Millipore filters. 100mg of the test sample in water: methanol (9:1) was used. To each extract, 5 ml of methanol was added**.** A 0.45-micron Millipore filter was used for filtering. A volume of 20µl of the sample was injected into the HPLC system.

For Quercitin: The column used was phenomex C18 (250x4.6,5µm), Detector 265, flow rate 1.2ml/mt, injection volume 20µl column, temperature 35°C, mobile phase: methanol: acetonitrile: buffer. **(**42.5:42.5:15%v/v/v), the pH was adjusted to 5 with orthophosphoric acid this was filtered, degassed, and sonicated before use.

Standard and Sample Preparation: 10 mg of standard quercetin was taken in 100ml of methanol, followed by sonification, and 0.45-micron Millipore filters were used for filtration.10 mg of sample extract was taken in 100ml of methanol followed by sonification and filtered using 0.45-micron Millipore filters.

Invitro biological activity [15]

a. DPPH Radical Scavenging Activity
The extracts were freshly prepared as stock solutions (10 mg/ml) in methanol before the start of the experiment. This stock solution was used to prepare the working solutions of suitable dilutions (20-140 µg/ml). DPPH was prepared as a 0.002 % solution in methanol. This was mixed with the sample extracts, followed by incubating in dark for ½ an hour, and the absorbance was measured at a wavelength of 517 nm. The DPPH solution without the sample solution was used as a control. The experiment was carried out in triplicate and the average calculated
The % absorbance is calculated using the formula:

$$\%\ inhibition = A - B \times 100/A \quad (4)$$

Where A - absorbance of the blank, B- absorbance of the sample

b. Hydrogen Peroxide Scavenging Activity
Various concentrations (50-300 µg/ml) of the test solutions were taken in test tubes, followed by the addition of 0.6ml hydrogen peroxide solution. Phosphate buffer (100 mM, pH 7.4) was added to make up the volume to 1 ml. The absorbance was measured at 230 nm. The phosphate buffer was taken as blank. Hydrogen peroxide scavenging activity is expressed as the percentage inhibition and was calculated using the following equation:

$$\%\ inhibition = A - B \times 100/A \quad (5)$$

Where A- absorbance of the blank, B - absorbance of the sample

Results and Discussion

Plant extracts are used traditionally by the majority of the world's population. The economic benefits trading of herbal medicines is boundless. Scientific validation for the medicinal herbs is however absent and is one of the hurdles in their use. Endogenous compounds of the plants are dissolved depending on the nature of the solvents and the process of extraction.

Solvent polarity is one of the decisive factors for extraction. Moreover, phytoconstituents present in plants may be polar, and some others non-polar in nature [16]. Phenolic compounds like flavonoids and phenolic acids are soluble in polar solvents mainly due to the availability of hydroxyl groups. Solvents having high polarity have the potential to extract compounds with a wider polarity allowing non-phenolics of polar nature i.e., carbohydrates and proteins to be dissolved [17]. Hence a mixture of ethanol and water (1:1) was preferred as the extracting solvent. The yield of the mature leaves was higher (36.2 gms) than that of the frontal leaves (24.7 gms). ML Ex and FL Ex were evaluated for various characteristics such as moisture content, total ash, alcohol soluble, acid insoluble ash, extractive value, and crude fiber were evaluated, and the obtained results are depicted in **Table 1**.

Table 1. Proximate analysis for mature and frontal leaves

Proximate analysis (n =3)	Mature	Frontal
Moisture content (%)	9.10±0.23	7.56±0.28
Total Ash content (% w/w)	6.56±0.37	4.13±0.39
Alcohol soluble ash	1.56±0.82	1.06±0.18
Acid insoluble ash	2.40±0.09	1.03±0.18
Water soluble extractive	10.5±0.12	9.16±0.18
Alcohol soluble extractive	19.2±0.36	12.4±0.12
Crude fibre (%)	10.4±0.43	7.86±0.32

Mean ±SEM, n = no. of a trial conducted.

Results have revealed that the moisture (9.1±0.23) and ash content (6.56±0.37) were higher in mature leaves when compared to the frontal leaves (7.56±0.28 and 4.13±0.39 respectively). This could be due to the presence of fibers, veins, and vein islets resulting in enhancing these contents. Apart from that, the proximate parameters also vary with the age and dimension of the leaves and the climatic conditions [18].

Flavonoids and phenolic acids play a significant role in activities like analgesic, skin protection anti-inflammatory, anti-oxidant, and anti-microbial activity as available in the literature [19, 20]. These compounds are good antioxidants hence we assessed the total phenolic acid and flavonoid content. Free radical scavenging activity is attributed to the Hydroxyl groups present in the plant. The Folin ciocalteu method is a commonly used method for the quantification of plant phenolics that may be present in the extracts. In this assay phenolic compounds under basic conditions react with the reagent forming chromogens, these chromogens can be easily detected at 750 nm. The results were obtained from a calibration curve (y = 0.0282x+ 0.013, R2 = 0.9961) of gallic acid and expressed in gallic acid equivalents (GAE) per gram dry extract weight. Total phenolic acid was found to be 290.66±0.85 µg/ml and 213.33 ±0.895 µg/ml for the ML Ex and FL Ex respectively. The total flavonoid content (TFC) of the extract was estimated by aluminum chloride colorimetric assay; quercetin was used as a standard flavonoid marker. The total flavonoid content was derived from the calibration curve (y = 0.0957x+ 0.0051, R^2 = 0.9967) of quercetin. The flavonoid content was 16.33 ±0.701 µg/ml and 11.33 ±0.895 µg/ml for the ML Ex and FL Ex.

Each value is the average of three analyses ± standard deviation

HPLC Analysis

The HPLC analysis of the two samples was performed by applying the conditions described earlier in the methodology section. Gallic acid and quercetin have emerged as strong antioxidants amongst phenolic acids and flavonoids. Hence they were selected as biomarkers in this study. The presence and variation in the amount of these compounds were identified by comparing the chromatographic peaks, retention time, and peak areas of the individual standards with the two samples. HPLC is a common, sensitive, and accurate method that has been widely used for the quality assessment of plant extract. Standard Quercetin showed a retention peak at 2.47; this was compared with the samples. **Figure 1** represents the HPLC chromatogram of quercetin for the ML Ex The ML Ex had a peak height of 276707 and the % area was 18.243 while the FL Ex chromatogram had a height area of 230391 and the % area was found to be 13.358 as depicted in **Figure 2**. Standard gallic acid exhibited a retention peak at 3.45. **Figure 3** represents peak for the phenolic acid i.e. gallic acid present in the ML Ex .ML Ex was having a height of 780114 and the % area was 97.892 while FL Ex had a height area of 607483 and the % area was found to be 93.478 as seen in **Figure 4**. From this observation, it was apparent that the amount of gallic acid and quercitin was more in the ML Ex when compared to FL Ex. The results of the HPLC further authenticated the difference in the amount of phytoconstituents in the frontal and mature leaves as seen by comparing the area under the curve. This is evidence that growth phases have an impact and have a major role in the production of plant constituents.

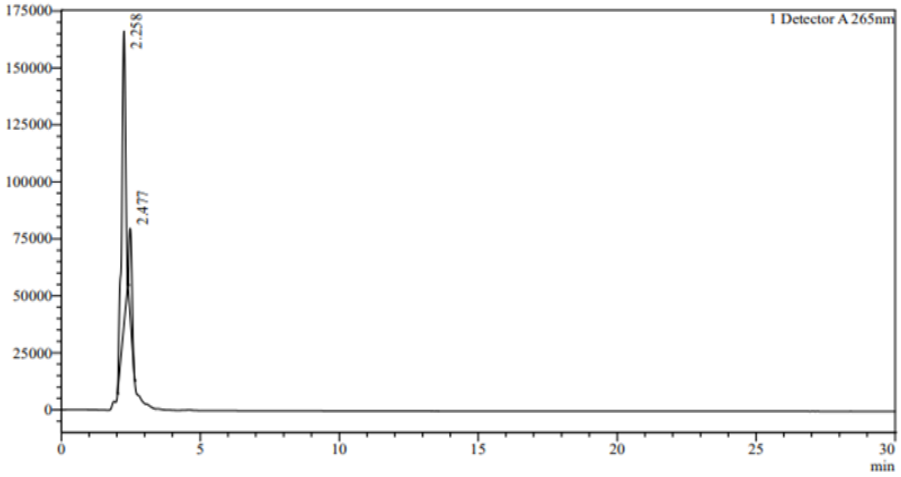

Figure 1. HPLC chromatogram for quercetin (ML Ex)

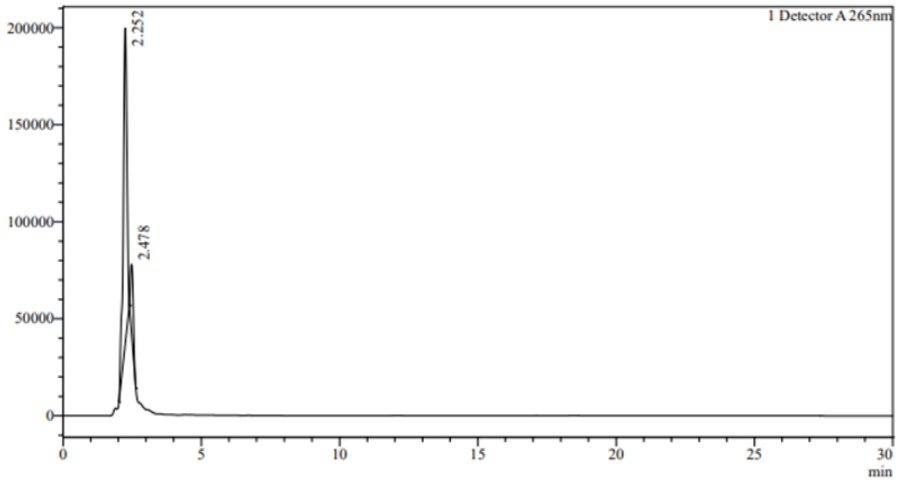

Figure 2. HPLC chromatogram for quercetin (FL Ex)

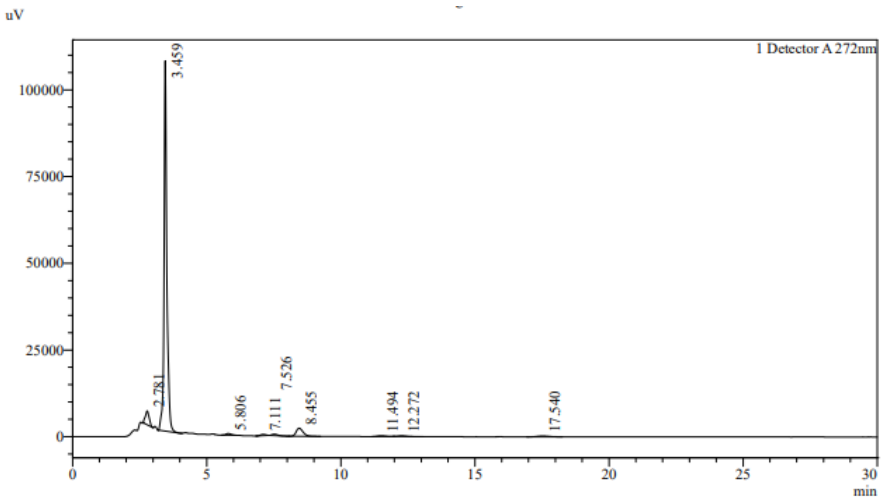

Figure 3. HPLC chromatogram for gallic acid (ML Ex)

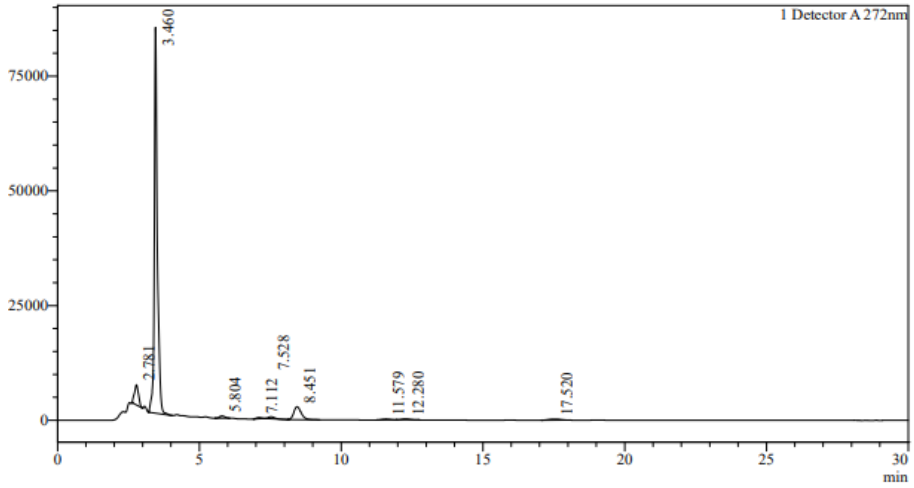

Figure 4. HPLC chromatogram for gallic acid (FL Ex)

Phenolic acids and flavonoids exhibit antioxidant activity under their ability to scavenge free radicals and inhibit the oxidative mechanisms that lead to various degenerative diseases. Polyphenols in plants behave as reducing agents and antioxidants due to the presence of the hydroxyl groups which impart hydrogen-donating properties and also by scavenging certain reactive species and suppressing lipid peroxidation recycling [21]. DPPH is one of the important methods for evaluating the free radical-scavenging activity of plant phenolics and serves as a substrate to evaluate the anti-oxidative activity. Anti-oxidants present in plants possess hydrogen atom donating capacity having the capability to quench DPPH radicals by transforming them into colorless products which bring about a reduction in absorbance [22]. The decrease in DPPH absorption indicates the capacity of the plant extracts to scavenge free radicals. Using this method, it is possible to determine the antiradical capacity by quantifying the reduction in the absorbance of DPPH in its radical form which disappears after accepting an electron or hydrogen radical from an antioxidant compound in the sample to become a stable diamagnetic molecule [23, 24]. **Figure 5** illustrates the antioxidant activity of the ML Ex and FL Ex by DDPH. Concentrations in the range of 20-140 µg/ml of the hydroalcoholic extracts were evaluated for antioxidant activity by this model. The recorded results revealed that the scavenging by the extracts was based on the concentration. The scavenging effect of the extracts and standard decreased in the following order: ascorbic acid >mature leaf extract > frontal leaf extract, which were 94.96%, 90.16 %, and 83.93% at 140 µg/mL respectively. Each value is the average of three analyses ± standard deviation.

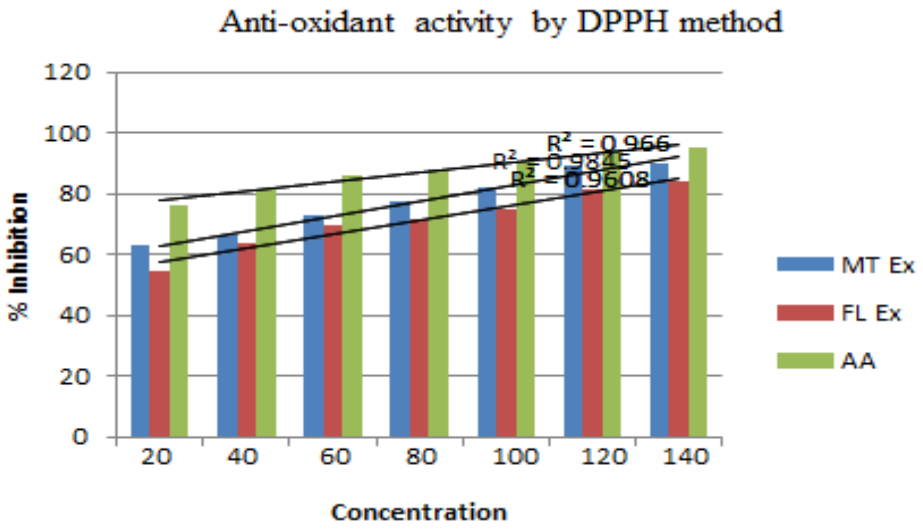

Figure 5. Antioxidant property of the extract by DPPH method

Hydrogen peroxide acts as a weak oxidizing agent that inactivates certain enzymes generally by oxidation of essential thiol groups. Hydrogen peroxide can be formed *in vivo* by an antioxidant enzyme such as superoxide dismutase. However it as such not very reactive, but sometimes can be harmful to cells as it may enhance OH. Hence removing H_2O_2 is very crucial for the protection of pharmaceuticals and food systems [25]. The ability of the extracts to scavenge hydrogen peroxide is indicated in **Figure 6**. The extract was capable of scavenging H_2O_2 in a concentration-dependent manner. At 150 µg/mL, the

activity was as follows ascorbic acid >mature leaf extract > frontal leaf extract i.e. 66.11%>52.45 %> 45.35 %. These results showed that the mature leaf showed better activity when compared to the frontal leaf extracts. However, the DPPH method showed better antioxidant activity when compared to this method. Each value is the average of three analyses ± standard deviation.

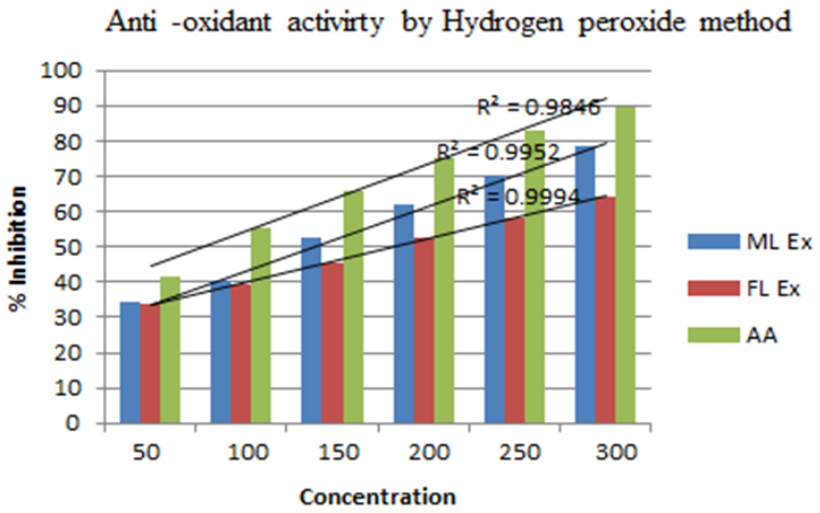

Figure 6. Antioxidant property of the extract by hydrogen peroxide method

The results recorded have shown a higher amount of phenolic acid and flavonoid in ML ex; a positive relationship between chemical compositions and the obtained results of antioxidant activity studies was noticed. It has been suggested that there is a strong positive correlation between total phenolic content and antioxidant activity [26]. In our study, it was found that the hydroalcoholic extract of mature leaves was rich in flavonoids and total phenolic content and these substances are well-documented for their antioxidant activity. These results were in harmony with previous findings which suggest that the various stages of development play a significant role in the formation of secondary metabolites as reported by other groups of researchers in the literature [27, 28].

Conclusion

The results of the study reveal that the ML ex has shown better antioxidant activity when compared to the FL ex by virtue of the difference in the number of phytoconstituents i.e., flavonoids and phenolic acids that have good antioxidant potential. The mechanism of action could be either via scavenging or chelation. This demonstrates that the stage of development of plant parts plays a significant role in the formation of secondary metabolites which in turn affect biological activities. However, more research may be required to ascertain the impact of growth stages on specific /various pharmacological activities of the plant to further confirm the findings of our study which may guide further medicinal investigations. Furthermore, this information could help in ascertaining the growth stage with the highest phytoconstituents and the harvesting of the plant at the appropriate stage

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