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Characterization of Bioactive Components of *Callistemon Lanculatues* by HPLC-DAD.

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Abstract

Callistemon lanculatues leaves and flowers contain active components. So this could be used as an antimicrobial and antioxidant component. This study aims to determine the chemical composition, and the best method for extracting total phenolic, total flavonoids, and antioxidant activity by DPPH to identify the most important uses of this plant. Six solvents were used according to differences in polarity: water, methanol, ethanol, ethyl acetate, chloroform, and petroleum ether respectively. Methanol extract showed the highest activity as antimicrobial, so it identified the active components of this extract by HPLC-DAD analysis. Results showed that the highest concentration of antioxidant activity in methanol extract was 40.9 %, while ethanol extract was 38.7% in the leaves. In flowers, methanol extract activity was 33.97%, while ethanol extract was 36.1%. Nine phenolic components were identified the highest amount quinic showed (0.1682 mg/ml). Followed by cinnamic (0.0192 mg/ml) in flowers, while in leaves quinic showed (0.0529 mg/ml) and cinnamic (0.0180 mg/ml). Flavonoid Kaempferol showed the highest concentration 533.52 mg/ml) followed by (Diosmin 258.127 mg/ml) in flower, while in leaves Kampferol showed (195.73 mg/ml) and Quercitin (118.73 mg/ml).

Key words:-*Callistemon lanculatues.* Antioxidant. HPLC analysis. Extraction.

Introduction

Due to its many different qualities, herbal medicines have been demonstrated to benefit people and have seen exponential growth in use in recent years *Callistemon. citrinus is also named as Callistemon lanceolatus*. Indigenous people in far-off places have used herbal medicine since antiquity, and it is often used in many developing nations. The use of synthetic chemicals has a significant impact on all living forms, causing an increase in the amplification of all life (**Kavitha and Satish, 2013).**

Aboriginal Australians depending on how their flowers appear, *callistemons* are also referred to as "bottle brushes." They can be found naturally along Australia's temperate east and south-west coasts **(Sumitra, 2014).** In traditional medicine, *callistemon* is valued for its ability to treat coughs, bronchitis, and insects, and its volatile oils have been utilized as antibacterial and antifungal agents. *callistemon* has a solid reputation as a medicinal plant and is highly useful in the treatment of specific illnesses**(Nash, 2000)**.

Sesquiterpene hydrocarbons were abundant in the plant's flower, followed by oxygenated monoterpenes, oxygenated sesquiterpene, and monoterpene hydrocarbons. Eudesmol, caryophyllene, bornyl acetate, eucalyptol, bicyclo germacrene, and 1R-Pinene were the primary elements that distinguished the blooms. Similar to this, the plant's stem is made up of monoterpene hydrocarbons and oxygenated monoterpenes. Eucalyptol, alpha-pinenes, limonene, and terpineol were the main substances predominating in the stem (**Kumar *et al*., 2018).**

Petroleum ether extract of *C. viminalis* leaves had a higher IC50 value of 56.2 and 0.54 g/mL for antioxidant capability when compared to industry standard compounds like butylated hydroxytoluene. The components of *C. viminalis* fruits and bark that showed the highest antioxidant activity were methyl gallate, gallic acid, catechin, and ellagic acid; additionally, the total extracts, petroleum ether, methylene chloride, and ethyl acetate fractions showed antioxidant activity that was comparable to that of ascorbic acid, the standard antioxidant **(Spencer *et al*. 1991)**.

An antioxidant supports an organism's defenses against free radicals and helps it delay or prevent oxidative damage. Numerous degenerative diseases are prevented by these antioxidants, such as cancers, neurological conditions, Alzheimer's disease, inflammatory diseases, and cardiovascular problems **(Kumaraswamy and Satish, 2008 and Karthaniah *et al*, 2011).** Due to being rich in polyphenols, researchers expected that *C.lanceolatus* would have anti-diabetic properties. Considering the plant's biological significance as an anti-inflammatory and anti-diabetic **(Kent *et al*., (2002) and Song *et al*, (2005)**.

Methanolic extracts of *C. lanceolatus and V. rosea* leaves and their combinations at various ratios greatly inhibited larval development as compared to methanolic extract controls **(Halder *et al*., 2010).** Antioxidants are frequently employed in fatty meals and oils to prevent oxidation **(Hegazi and El-Lamey, 2011).** The goal of this investigation is to naturally investigate the best extraction technique for useful materials and the chemical composition of *Callistemon* plants in flowers and leaves, as an estimation of the active vehicle activity in extracts, and the best solvent for extracting the antioxidant of extracts by DPPH. Investigation of the phenolic component profile using HPLC of the ethanolic extract of flowers and leaves.

Materials and Methods

Materials:

Callistemon lanceolatus (fresh flowers and leaves) were collected from faculty of agriclture; Benha University. Qaliobia governorate, Egypt. Flowers and leaves are washed and left in a clean place in the shade away from light and heat until complete dryness and ground into powder by using a waring blender for further investigation.

Preparation of extracts:

Exactly fifty grams of leaves or flower powder of *C. lanceolatus* were extracted by soaking with water, methanol, ethanol, ethyl acetate, chloroform, and petroleum ether for 24 hours. The different extracts were saved in clean dark-colored bottles after concentration under reduced pressure using a rotary vacuum evaporator. To identify the most suitable solvent for the extraction of total phenolic, total flavonoids, and antioxidant activity.

Analytical methods:

Moisture, ash, crude protein, and total lipids were determined according to the method of the Association of Official Analytical chemists **A.O.A.C. (2019).**

Determination of total carbohydrates:

The concentration of total carbohydrates was determined according to **(Chaplin, 1994).**

Determination of total phenolic.

The total phenolic analysis content of the extracts was determined by spectrometry method **(Muntana and Prasong, 2010)** 100 µL sample was added with 1 mL Folin-Ciocaiteu 10% and 2 mL Sodium Carbonate 7.5%. The mixture was added with water in a 10 mL volumetric Flask and shaken. The solution was incubated at ambient temperature for 30 min and the absorbance of the sample was measured at absorbance 760 nm. The Total phenolic content of the sample was stated by gallic acid equivalence (GAE)/g sample on dry base.

Determination of total flavonoids.

The amount of total flavonoids in the extracts was measured by the method of **Tandoro *et al*, (2020).** 200 µI sample was added with 0.3m $NaNO_2$ 5% (b/v),3.0 ml $AlCl_3$ 10% (b/v), and 2 ml NaOH 1 M in a 10 mL volumetric flask. The mixture was shaken and diluted with water until a volume of 10 mL. The absorbance of the sample was measured at 510 nm. The total flavonoid content of the sample was stated by Quercetin equivalence (QE)/g sample on dry bas.

Determination of antioxidant activity by DPPH :

The antioxidant activity of *Callistemon* flowers and leaves extracts was measured by using the method of **Tandoro *et al*., (2020).** 3 mL of DPPH solution (4 mg /100 mL mixture was incubated at ambient temperature for 30 min. The absorbance of the sample was measured at 517 nm. Percentage of Antioxidant activity of free radical DPPH was calculated as follows:

Antioxidant activity (inhibition) % = [(A control - A sample) / A control] × 100

Where **A control** is the absorbance of the control reaction and **A sample** is the absorbance of the plant extract.

HPLC analysis

The separation for each of *Callistemon* leaves and flowers ethanolic extracts was carried out using the system Thermo (Ultimate 3000) consisting of a pump, automatic sample injector, and associated DELL-compatible computer supported with Cromelion 7 interpretation program. A diode array detector (DAD)-3000 was used. The Thermo-hypersil reversed phase C18 column 2.5× 30cm was operated at 25º C. The mobile phase consists of 0.05%Trifluoroacetic acid/Acetonitrile (solvent A) and distilled water (solvent B). The UV absorption spectra of the standard as well as the samples were recorded in the range of 230–400 nm. Samples and standards solutions as well as the mobile phase were degassed and filtered through a 0.45 µm membrane filter (Millipore). Identification of the compounds was done by comparison of their retention's time and UV absorption spectrum with those of the standards. Inj. Vol: 20 µL; Column: RP- C18; Column size: 2.5× 30cm; Mobile phase: 0.05%Trifluoroacetic acid/Acetonitrile / H_2O **(Biswas., *et al* 2013).**

Results and Discussion

Chemical composition of *callistemon lanceolatus* leaves and flowers.

The chemical composition of *C.lanceolatus* is shown in Table (1). The results revealed that flower contained moisture (10.04g/100 g), Ash (5.43g/100g), protein (2.91g/100g), (68.4g/100g) of total carbohydrates and lipids (8.85 g/100g). While the leaves contained moisture (7.59g/100 g), Ash (5.64g/100g), protein (2.14g/100g), (71.6g/100g) of total carbohydrates, lipids (7.7g/100g). There is a scarcity of information about the chemical composition file so the current study aims to investigate basic chemical components, in addition to antioxidants and phenolic content for *Callistemon*. Moisture content (3.4%), total ash in the leaves (4.66%). This results were in agreement with those reported By **(El Dib and El-Shenawy., 2008) and (Chistokhodova *et al.*, 2002)**.

Table 1. Chemical composition of *callistemon lanceolatus* leaves and flowers (mean±SD).

Components	Contents(g/100g) of Flowers	Contents(g/100g) of Leaves
Moisture	10.04±0.1	7.59±0.2
Ash	5.43±0.3	5.64±0.04
Crude protein	2.91±0.2	2.14±0.05
Total carbohydrates	68.4±0.4	71.6±0.3
Total lipids	8.85±0.2	7.7±0.1

Value are mean of three replicates ± standard deviation (n=3)

Total Phenolic, Total flavonoid compounds and Antioxidant activity of *Callistemon lanceolatus* extracts of *Callistemon* flowers :

Data in Table (2), indicate that flower ethanol extract (67.8 w/w) had the highest ratio. While chloroform extract (7.51w/w) had the lowest ratio. Water was the most efficient technique for extracting total polyphenols followed by ethanol and methanol (1.997,1.73 and 1.72 mg GAE/100g). Data concerning total flavonoid content showed that ethanol, methanol, and ethyl acetate had almost the same ratios (0.22, 0.21, and 0.21 mg QE/100g). Ethanol and methanol extracts showed the highest antioxidant activity in Callistemon flowers (36.1 and 33.97%) by DPPH while chloroform extract had the lowest activity. *Callistemon citrinus* is one of the species with the greatest therapeutic potential and capacity to tolerate pollution. The species were shown to be abundant in bioactive chemicals, such as various polyphenols (phenolic acids and flavonols)**(Radulović *et al.*, 2015).**

Table 2. Total phenolics, total flavonoids, and antioxidants activity of *callistmon* of flowers.

Parameters	Distilled water	Methanol	Ethanol	Petroleum ether	Chloroform	Ethyl acetate
Yields (w/w) %	17.8±0.02	34.7±0.03	67.8±0.03	10.95±0.02	7.51±0.02	10.4±0.02
Total polyphenols (mg GAE/100g)	1.997±0.02	1.72±0.012	1.73±0.02	1.27±0.02	0.40±0.02	1.52±0.02
Total flavonoids (mg QE/100g)	0.18±0.015	0.21±0.01	0.22±0.02	0.07±0.03	0.14±0.01	0.21±0.015
Antioxidant activity(%)by DPPH	6.38±0.15	33.97±0.1	36.1±2.22	19.85±0.15	1.40±0.1	15.58±0.1

Value are mean of three replicates ± standard deviation (n=3)

Total Phenolic, Total flavonoid compounds, and antioxidant activity of *Callistemon* extract of leaves.

Results in table (3), show that methanol extract of leaves (65.55 w/w%) had the highest yield ratio. While, ethanol extract (10.68%). Methanol was the most efficient technique for extraction total polyphenols (1.42 mg GAE/100g) followed by petroleum ether (1.3 GAE/100g). Total flavonoid content revealed the ethanol extracted the highest flavonoid content followed by chloroform and ethyl acetate (1.3, 0.77 ,and 0.77 mg QE/100g). Antioxidant activity % results indicated that methanol and ethanol had the highest values (40.9 and 38.69 %) while, distilled water value had the lowest ratio 8.62% by DPPH. The obtained results are in agreement with those reported by **(Hasan *et al.*, 2016; Mazumder *et al.*, (2022).** The phenolic content of ethanol extracts (80%) made from the leaves and cell cultures of the three species of *Callistemon* was standardized. The maximum concentration of phenolic (104 2.0 mg-g-1) was found in the leaves of *C. lanceolatus* **(Abdelhady *et al.*,2011).**

Table 3. Total phenolics, total flavonoids, and antioxidants activity of callistmon of leaves.

Parameters	Distilled water	Methanol	Ethanol	Petroleum ether	Chloroform	Ethyl acetate
Yields (w/w) %	38.20±0.03	65.55±0.02	10.68±0.03	37.21±0.04	14.55±0.02	15.9±0.04
Total polyphenols (mg GAE/100g)	1.27±0.03	1.42±0.02	0.21±0.04	1.38±0.03	0.21±0.02	1.22±0.096

Total flavonoids (mg QE/100g)	**0.08±0.02**	**0.13±0.015**	**1.3±0.02**	**0.1±0.01**	**0.77±0.096**	**0.77±0.096**
Antioxidant activity(%)by DPPH	**8.62±0.13**	**40.9±0.1**	**38.69±1.0**	**23.2±0.13**	**18.6±0.15**	**18.6±0.15**

Value are mean of three replicates ± standard deviation (n=3)

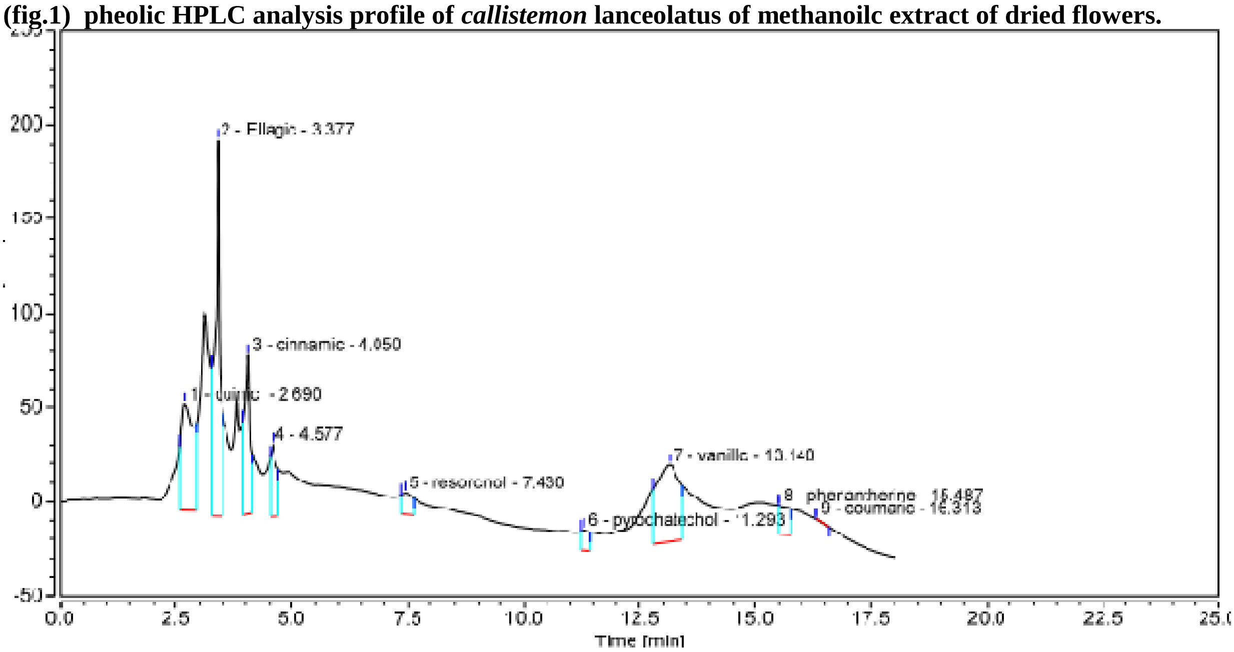
Identification of some phenolic and flavonoid components of *Callistemon lanceolatus* methanolic extract by HPLC -DAD in flowers and leaves:

Data in tables (4) figures (1), and (2) reveal the isolation and identification of seven phenolic compounds in flowers. Quinic showed the highest concentration (0.1682 mg/mL). followed by cinnamic (0.0192 mg/mL), **and** resorcinol (0.0007 mg/mL). Ellagic (0.0168 mg/mL). Pyrocatechol, vanillic, and phenantherine showed the same concentration (0.0003 mg/mL). Nine phenolic compounds were identified in leaves quinic (0.0529 mg/mL) and cinnamic (0.0180 mg/mL) showed the highest concentration while vanillic and ferulic showed the lowest concentration (0.0001 mg/mL). The varying effectiveness of extracting solvents in dissolving endogenous compounds varies depending on the age of the tree, as well as depending on the season of cultivation and the extraction process. This might be related to environmental variables such as the varying ecological and meteorological conditions in the locations, the kind of plant, and the manner of processing **(Ogundajo *et al.*, 2013).** Quinic is the most concentrated in compounds, this acid is a versatile chiral starting material for the synthesis of pharmaceuticals.

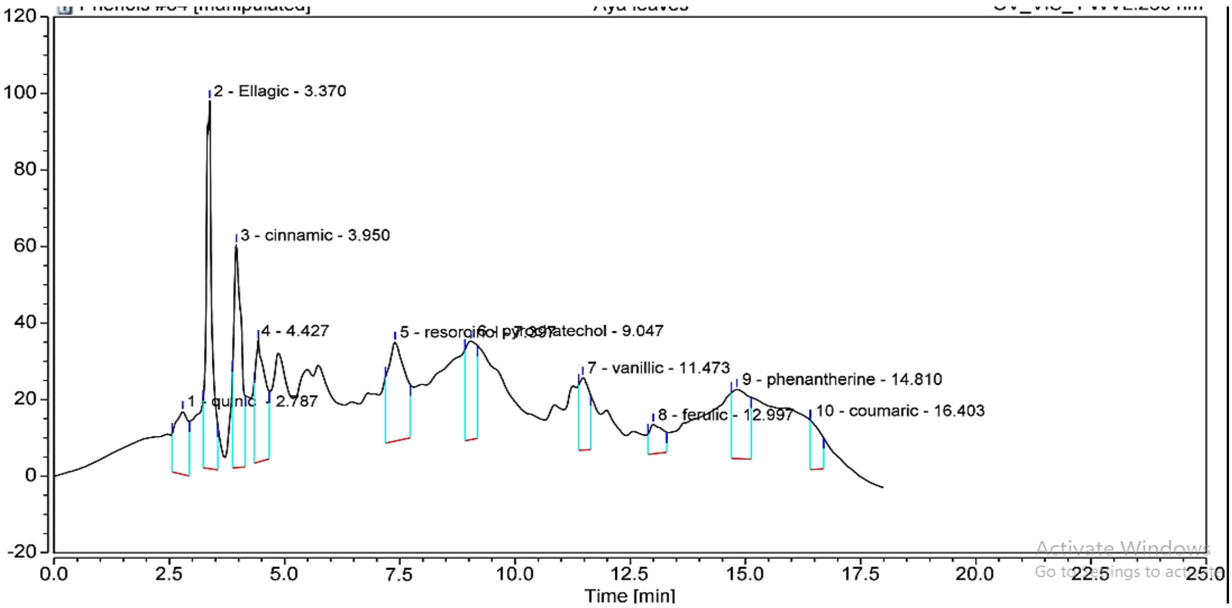
Table 4. Pheolic compounds of *callistemon lanceolatus* of flowers and leaves extract analyzed by HPLC- DAD.

	Flowers		**Leaves**	
Components	**Concentration Mg/mL**	**Retention Time min**	**Concentration Mg/Ml**	**Retention Time min**
1. quinic	**0.1682**	**2.690**	**0.0529**	**2.787**
2. Ellagic	**0.0168**	**3.377**	**0.0096**	**3.370**
3. cinnamic	**0.0192**	**4.050**	**0.0180**	**3.950**
4. resorcinol	**0.0007**	**7.430**	**0.0027**	**7.397**
5. pyrochatechol	**0.0003**	**11.293**	**0.0008**	**9.047**
6. vanillic	**0.0003**	**13.140**	**0.0001**	**11.473**
7. ferulic	**0**	**0**	**0.0001**	**12.997**
8. phenantherine	**0.0003**	**15.487**	**0.0006**	**14.810**
9. coumaric	**0**	**0**	**0.0089**	**16.403**

(fig.1) pheolic HPLC analysis profile of *callistemon* lanceolatus of methanoilc extract of dried flowers.



(fig.2) pheolic HPLC analysis profile of *callistemon* lanceolatus of leaves of methanoilc extract of dried leaves.



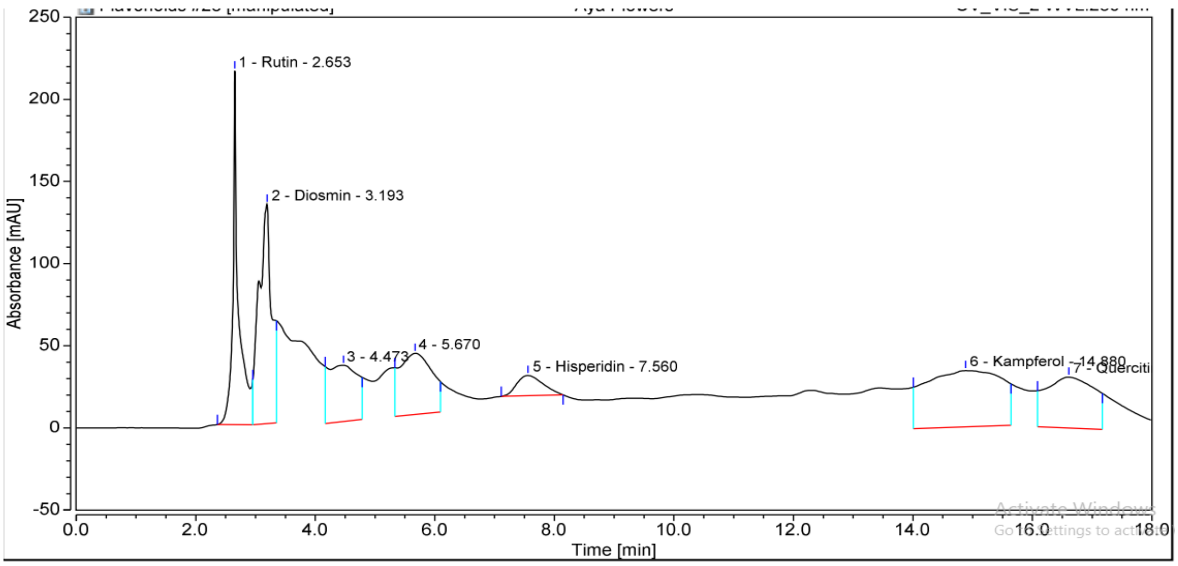
Identification of some phenolic and flavonoid components of *Callistemon lanceolatus* *Callistemon lanceolatus* methanol extract by HPLC - DAD in leaves:

Data in table (5) and figures (3). (4) revealed the isolation and identification of five flavonoid compounds were found in flowers. Kampferol (533.52 mg/mL). Diosmin (258.127 mg/mL), Quercitin (171.58 mg/mL), Hisperidin (55.298 mg/mL) and Rutin had the lowest concentration (10.65 mg/mL). Four flavonoid compounds were found in the leaves. Kampferol (195.92 mg/mL), Quercitin (118.73 mg/mL), Rutin (118.73 mg/mL) and Diosmin (17.7 mg/mL). These results were in agreement with those reported by**(Bhatia *et al.*, 1972), (El Dib and El-Shenawy., 2008),** Goyal *et al.*,(2012), **(Khubeiz and Mansour., (2016).** Kampferol had the highest concentration, it works as an antimicrobial prevention and treatment of human immunodeficiency virus and other infectious diseases followed by Diosmin is a chemical in some plants, It's found mainly in citrus fruits. Diosmin is most often used for hemorrhoids and leg sores caused by poor blood flow.

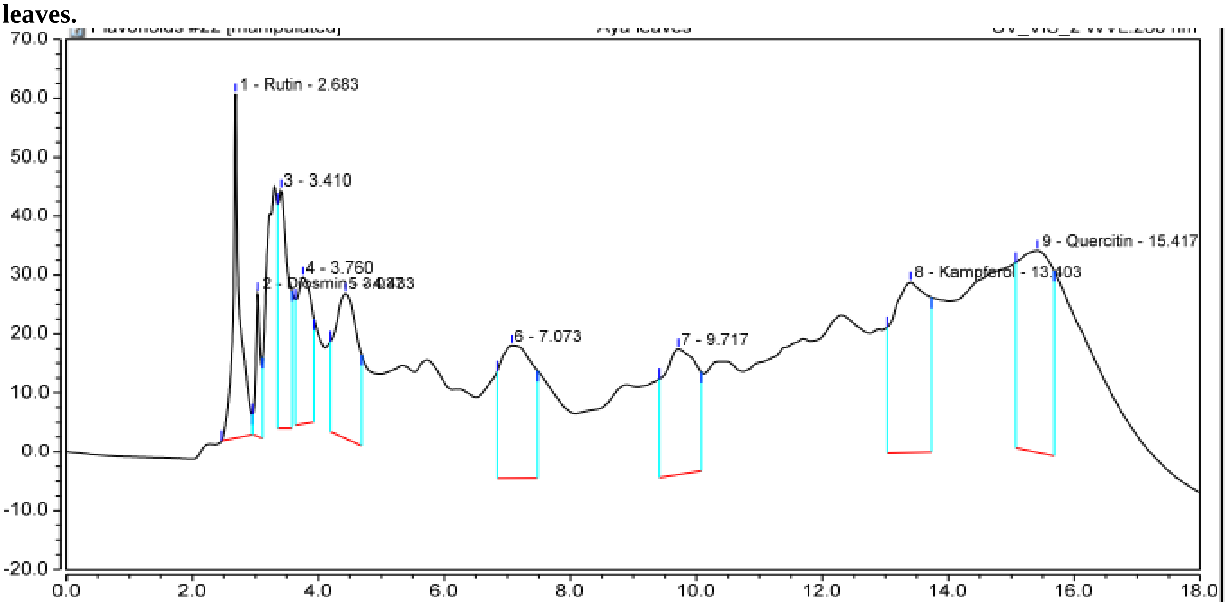
Table 5. Flavonoid compounds of *callistemon lanceolatus* of flowers and leaves extract analyzed by HPLC-DAD.

Components	Flowers		leaves	
	Concentration Mg/mL	Retention Time min	Concentration Mg/Ml	Retention Time min
1. Rutin	10.65	2.653	3.1172	2.683
2. Diosmin	258.127	3.193	17.6992	3.037
3. Hisperidin	55.298	7.560	0	0
4. Kampferol	533.52	14.880	195.92	13.403
5. Quercitin	171.58	16.610	118.73	15.417

(fig.3) Flavonoid HPLC analysis profile of *callistemon* lanceolatus of methanoilc extract in dried flowers.



(fig.4) Flavonoid HPLC analysis profile of *callistemon lanceolatus* of leaves of methanoilc extact in dried leaves.



Conclusion

This study confirms that the phenol extract and all extracts from *Callistemon lanceolatus* (flowers and leaves) could be an important source of bioactive compounds. Methanol, ethanol, and water showed the highest ratio for extraction yield, polyphenols, and Flavonoids while antioxidant activity methanol extract showed the highest ratio. Some phenolic and flavonoids compounds were identified by HPLC-DAD as kampfero, hisperidin, diosmin, and rutin. Quercetin was identified in flowers. pyrocatechol, cinnamic, resorcinol, and ellagic. The leaves component contained phenanthroline. There was phenanthroline in the leaf component.

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تصنيف بعض مركبات مضادات الاكسدة لنبات الكالستيمون باستخدام HPLC-DAD.

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ان نبات الكالستيمون المسمي بنبات فرشاة الزجاج والذي ينتشر في مصر كنبات للزينة غني بمضادات للاكسدة التي توجد في الاوراق والازهار حيث تم دراسة الخواص الكيميائية وتوصيف المكونات الفعالة ومضادات الاكسدة التي يحتوي عليها كلا من اوراق وازهار نبات الكالستيمون تم تقدير المكونات الكيميائية الاساسية وتقدير المكونات الحيوية الفعالة وتقدير نشاط المستخلصات الفينولية كمضادات اكسدة طبيعية حيث يتم استخدامها في الطب التقليدي لعلاج العديد من الامراض المزمنة مثل ضيق التنفس وغيرها والحفاظ علي الصحة بشكل جيد

أوضح التركيب الكيميائي الازهار بعد تجفيفها لنبات الكالستيمون والذي يسمي بفرشاة الزجاج ان الازهار تحتوي علي 10.04 % رطوبة و 5.43 % رماد و 2.91 % بروتين و 68.4 % كربوهيدرات و8.85 % دهون .

أوضح التركيب الكيميائي في الاوراق بعد تجفيفها انها تحتوي علي رطوبة 7.59% ورماد 5.64 % و بروتين 2.14% و كربوهيدرات 71.6% و دهون 7.7%.

وباستخدام ستة مذيبات مختلفة وهم الماء والإيثانول والميثانول وخلات الإيثيل و الكلوروفورم و الاثير البترولي وتمت تقدير المركبات الفينولية والفلافونويدات وتحديد افضل طريقة لاستخلاص المركبات الفعالة في الازهار والاوراق حيث اثبتت النتائج ان النبات يحتوي علي مركبات غنية بمحتواها الفينوليو مركبات الفلافونويدية حيث تعمل كمضادات اكسدة طبيعية.

وباستخدام التحليل الكروماتوجرافي السائل للمستخلص الميثانولي ومن هذه المركبات الهامة حمض الجالك والسينامك والفانيلك و الكامبفيرول وغيرها من المركبات والاحماض الهامة .

حيث اعطي الميثانول افضل طريقة استخلاص والايثانول الايثير البترولي والمياة المقطرة و خلات الايثيل والكلوروفورم .

الكلمات الدالة :– الكالستيمون لينسيولاتس . التحليل الكروموتجرافي السائل. مضادات الاكسدة .فرشاة الزجاج.