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Chemical Composition and Biological Activities of *Marrubium vulgare L*

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Abstract: The present study was conducted to evaluate the antioxidant and antibacterial activity of the extract of leaves and flowering of *Marrubium vulgare L*. The quantification of total phenols was determined using the Folin-Ciocalteu reagent, it is 2.23 mg EAG/g DM. The identification of flavonoids by the Alcl3 method, revealed the presence of anthocyanins, C-glycosides and aglycones, whose contents are 1.266mg/g, 0.385mg/g and 0.0331 mg/g, respectively. The antioxidant activity of the compounds in the methanolic extract was evaluated in vitro through two tests. In the case of DPPH test, the IC50 are 0.55, 0.97, and 1.2, 0.033 mg/ml for anthocyanins, c-glycosides, polyphenols and aglycones respectively. For FRAP assay, at a concentration of 1 mg/ml, the reducing power of iron manifested ODs of 1.389 nm; 0.752 nm, 0.496 and 0.114 nm for anthocyanins, C-glycosides, total polyphenols and aglycones respectively. The results show an excellent correlation between the two tests used, and the contents of anthocyanins and c-glycosides. The antimicrobial activity was evaluated by the agar diffusion method against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922). The antibiogram shows that anthocyanins have a very important antibacterial activity on *S. aureus* with an inhibition zone of 16.5 mm and a diameter of 13.5 for *Escherichia coli.* Anthocyanins show the lowest MIC values against the two strains *Staphylococcus aureus* and *Escherichia coli* (MIC=1/16 and 1/8 respectively). C-glycosides also have an inhibitory effect on the growth of *Escherichia coli* and particularly on *Staphylococcus aureus* (MIC= 1/32 and 1/8 respectively) which is a sensitive bacterium (gram+). Anthocyanins have marked a strong sensitivity towards the two strains. In conclusion, this work presents the compounds of *Marrubium vulgare* L as novel antimicrobial agents and as a potential source for combating bacteria, which are increasingly resistant to antibiotics.

Keywords: Antimicrobial activity. Antioxidant activity, Chemical composition, Methanolic extract, *Marrubium vulgare* L.

Oxidative stress, the cause of many diseases, is driving the search for new antioxidant remedies. For a hundred years, synthesis had defeated plant-derived drugs, except for antibiotics and anti-tumor drugs. However, development of molecular level assays, are opening up for natural products. Similarly, biotechnological, biochemical and metabolic advances are leading to a rapidly growing interest in natural resources (Reena and al 2021). This interest is also due to the realization that a significant number of secondary metabolites, which play an important role in the prevention of diseases, are present in our food (Uttara 2009, Gowhar Amin Mir and Sandeep Sehgal 2021)

The researchers also reported on the antimicrobial activity of several medicinal plants due to the emergence of multi-resistant bacteria as they are a major cause of treatment failure in many infectious diseases. Therefore, there is a need to search for alternative antimicrobial agents. One of the possible strategies to achieve this goal is to identify and characterize bioactive phytochemicals, which have antibacterial activity (Ahanjan et al 2008). *Marrubium vulgare L*. is an important medicinal plant that belongs to the Lamiaceae family, common in Mediterranean regions. Rich mainly in phenolic compounds is known for its strong antioxidant and antimicrobial power (Casanova and Tomi 2018).This family has been used for centuries as an expectorant, liquefier of bronchial secretions, antitussive, anti-infectious, stomachic, diuretic, tonic, cholagogue, anti-hepatobiliary, febrifuge (Dusser Lauge 2017).

For all these reasons, present study is in line with the perspective of valorization of local natural resources. In this context research is focused on the identification of phenolic compounds and the evaluation of the antioxidant and antimicrobial activity of the methanolic extract of the leaves and flowers of *Marrubium vulgare* L. against 2 gram positive and gram negative bacteria.

MATERIAL AND METHODS

The species *Marrubium vulgare L*.,was collected during April to May 2018, in Makouda in the region of TiziOuzou (Algeria) far from any pollution impact. The leaves and

flowers of the plant were, dried in a well-ventilated place and protected from light, crushed and stored in glass bottles in the dark.

Study area: The wilaya of Tizi-Ouzou is located on the central-eastern coast of Algeria and covers an area of 2958 km2. It is limited to the South by the wilaya of Bouira, to the East by Bejaia, to the West by Boumerdès and opens to the North on the Mediterranean Sea by 85 km of coasts. The study area is located in the central part of the Tellian Atlas, in the North of Algeria, at a distance of 100km East of Algiers and West of the Djurdjura chain, between latitudes 36°20'N and 36°40'N and longitudes 3°40'E and 4°35'E.

Preparation of the methanolic extract: 10g of leaf and flower powder are macerated in 100 ml of methanol under stirring for 24 hours at room temperature. After filtration and evaporation of the solvent using a rotary evaporator, at 55°C, the extract is recovered with 5ml of methanol and stored in the refrigerator at 4°C (Djahra 2014).

Determination of total phenolic compounds: The content of total phenols was determined with the Folin-Ciocalteu reagent according to the technique of Singleton (1999) using gallic acid as standard. This determination is based on the quantification of the total concentration of hydroxyl groups present in the extract. The Folin-Ciocalteau reagent is an acidic yellow solution containing a polymeric complex of ions (heteropolyacids). In alkaline medium, this reagent oxidizes phenols into phenolate ions and partially reduces its heteropolyacids resulting in the formation of a blue complex. The absorbance of the samples and standards is measured with a spectrophotometer at 765 nm. The total phenol content is given in mg gallic acid equivalent (GAE)/g dry matter.

Determination of flavonoids: The flavonoids were estimated according to the method described by Hertog et al (1992) using quercetin as standard. The flavonoids are quantified by a colorimetric method with aluminum trichloride ($AlCl_3$) and soda (NaOH). Aluminum trichloride forms a yellow complex with flavonoids and soda forms a pink complex. The absorbance of the samples and standards is measured with a spectrophotometer at 430nm. The flavonoid content is expressed per mg quercetin equivalent/g dry matter.

Fig. 1. Location of the sample collection area (https://www.viamichelin.fr/web/Cartes-plans/Carte_plan-Tizi_Rached-_-Tizi_Ouzou-Algerie

Determination of proanthocyanins: The reading is made by measuring the absorbance at 520 nm. The absolute content of anthocyanins is calculated by the following formula with a corrective coefficient of 6 (Lebreton et al 1967):

$$T\ (mg/g) = 5{,}2 \cdot 10^{-2} \cdot DO \cdot V \cdot d/p$$

Determination of C-glycosylflavones: The reading is made by measuring the absorbance at 340 nm and the absolute content is calculated by the following formula:

$$T\ (mg/g) = 2{,}37 \cdot 10\text{-}2 \cdot DO.V.d/p$$

Determination of flavonicaglycones: To determine the content of aglycones, the differential assay method was used, which is based on two dilutions:

The 1st dilution is done in ethanol (ethereal extract + 95° ethanol). The 2nd dilution is done in AlCl3 solution (Ethereal extract + AlCl3 in 95° ethanol). The AlCl3 solution is prepared by mixing 1g of aluminum chloride in 100ml of 95° ethanol. From the dry ethereal residue taken up in 95° ethanol, 1ml of the 1% aluminum chloride solution is added. After incubation for 15 min at room temperature the absorbance of the aglycones was read between 400 and 435nm with a spectrophotometer. Concerning the determination of flavonicaglycones (420nm) and flavonols (435nm), the formula used is the following:

$$T(mg/g^{-1}) = 1{,}3 \cdot 10^{-2} \cdot \Delta DO \cdot V \cdot d/p$$

Evaluation of the antioxidant power of the extract

DPPH free radical scavenging test: The scavenging activity of the DPPH radical is measured according to the protocol described by Sanchez-moreno (2002). The solution of DPPH was prepared in advance by solubilizing 1.2mg of DPPH in 50 ml of methanol. To 1 ml of DPPH is added 0.5 ml of the extract solution at different concentrations. The mixture is kept in the dark for 30 min until decoloration. The absorbance is read against a blank prepared for each concentration at 517nm.

The positive control is represented by a solution of a standard antioxidant,ascorbic acid. The negative control (blank) consists of DPPH and methanol.

The test is repeated three times and the results are expressed as percentage reduction of DPPH (%) according to the following formula:

$$I\ \% = [1 - (Abs\ contrôle - Abs\ test)/ Abs\ contrôle] \times 100$$

The values of the concentrations to inhibit 50% of the initial concentration of DPPH (IC50) were determined graphically by linear or logarithmic regression of the percentages of inhibition as a function of the different

concentrations of the tested compounds (Torre et al 2007).

Iron reduction test FRAP: The experimental protocol of Yildirim (2001) was used 1ml of the extract at different concentrations is mixed with 1.25ml of a 0.2 M phosphate buffer solution (pH= 6.6) and 1.25ml of a 1% potassium ferricyanide K3Fe (CN)6 solution. The whole is incubated in a water bath at 50°C for 20 min, then cooled to room temperature. 2.5 ml of trichloroacetic acid (10%) was added to stop the reaction, The mixture was centrifuged at 4000rpm for 20 min at room temperature. To 1 ml of supernatant 1 ml of distilled water and 200µl of a 0.1% iron chloride (FeCl3, 6H2O) solution was added. Solutions of reference antioxidants (Ascorbic acid) that serve as positive control and as well as the negative control (blank, with methanol) were prepared under the same conditions. The OD of the test compounds and ascorbic acid were measured spectrophotometrically at a wavelength of 700 nm.

Determination of antibacterial activity: Two reference bacterial strains from the Pasteur Institute (Algeria) are tested: *Escherichia coli* ATCC 25922 Gram negative and *Staphylococcus aureus* ATCC 25923 (Gram positive).

From young colonies, from 18 to 24 h, a bacterial suspension is made in sterile distilled water for each strain. After homogenization of the bacterial suspension with a vortex, standardization to 106 CFU/ml was performed by spectrophotometer at a wavelength of 620nm. The OD obtained is between 0.08 and 0.1 which corresponds to a concentration of 107 to 108 CFU/ml (CFU: Colony Forming Unit).

Diffusion method on solid medium: antibiogram: The evaluation of antibacterial activity was performed by the agar diffusion method (Ngameni 2009). In Petri dishes, Mueller Hinton agar medium in supercooling was aseptically poured at a rate of 15ml per dish. After solidification, a sterile swab was soaked in the bacterial suspension and spread on the surface of the agar three times, rotating the plate at about 60° after each application in order to have an even distribution of the inoculum. Sterile discs impregnated with each sample tested (10µl per disc) were placed on the agar surface. The plates were then incubated for 24 h at 37 °C. Negative control discs impregnated with an aqueous solution of antibiotic (positive control) were included in the tests. The experiment was performed in triplicate. The diameters of the inhibition zones surrounding the discs containing the test samples were measured **(**Sharififar et al 2007). The solid-state diffusion technique (CMI) was used. It is a method similar to the antibiogram method which consists in determining the sensitivity of a bacterial strain towards one or several metabolites at different dilutions. The MIC is defined as the lowest concentration capable of inhibiting any microbial growth visible to the naked eye. The statistical study is performed with EXCEL software. The results are repeated 3 times and are expressed as mean ± SD.

RESULTS AND DISCUSSION

Total polyphenol content: The content of phenolic compounds estimated using a linear calibration curve (Fig. 2). The total polyphenols in the methanolic crude extract of *Marrubium vulgare L* was 2.23mg gallic acid equivalent per g dry matter. A rather close value (3.42mg GAE/g extract) was observed in the methanolic extract of leaves and flowers of *Marrubium vulgare L* by Ghedadba (2014).

On the other hand, different results were obtained from the vegetative organs of the same species. A level of 18, 21mg EAG/ml of extract was found by Boudjelal (2012) and 17.08 mgEAG/gMS by Djahra (2014). Stanković (2011) observed 49.27 mg GA/g extract in total phenols on a lamiaceae *Marrubium peregrinum L*, levels that seem high compared to present extract (2.23±0.114). This variability in the results could be related to the climatic conditions of the species' biotope or to the different methods followed during extraction (Stanković, 2011).

Flavonoid content: The anthocyanins represent the most important class of flavonoids, with a value of 1.266 mg/g. C-glycosides and aglycones present at lowest values of 0.385 mg/g and 0.0331mg/g respectively (Table 1). These flavonoid contents can be affected by genotype, development and growth conditions, maturity and by the

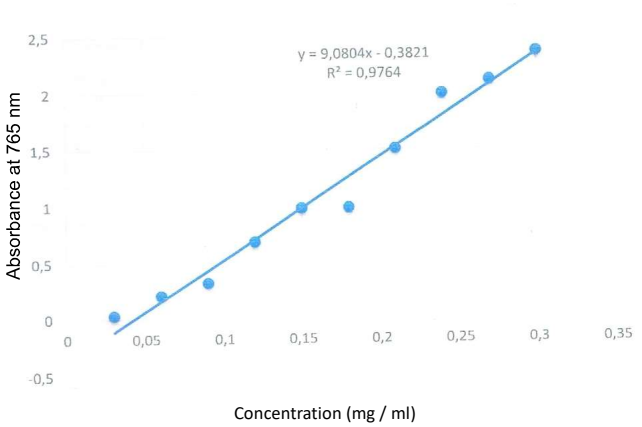


Fig. 2. Standard curve of gallic acid for the determination of total polyphenols

Table 1. Contents of the three classes of flavonoids

Phenolic compound	Content (mg. g^{-1})
Anthocyanins	1.266
C-glycosides	0.385
Aglycones	0.0331

polarity of the solvents used in the preparation of extracts (Ghedadba 201 and Bouterfas et al 2016)

Antioxidant activity

DPPH test

The percentage of free radical inhibition is proportional to the concentration of the compounds. Anthocyanins seem to have a better antioxidant activity than total polyphenols and c-glycosides. The aglycones represent the least efficient compounds in the elimination of free radicals. The antiradical activity of compounds was significantly lower than that of ascorbic acid (Fig. 3). To better characterize the antioxidant power, introduced the parameter IC50. This parameter expresses the quantity of antioxidants necessary to decrease the concentration of the free radical by 50%. The lower the IC50 value, the higher the antioxidant activity of a compound, which indicates the effectiveness of the compound (Hebi et Eddouks 2016)).

Anthocyanins and c-glycosides show antiradical properties with IC50= 0.55 and 0.97 mg/ml respectively. Total polyphenols show very low antiradical properties with an IC50 of 1.2 mg/ml. IC50 of aglycones is not determined and is probably due to its low content in the extract. The IC50 of ascorbic acid is a powerful anti-radical being 0.2 mg/ml (Fig. 4).

FRAP test

The iron reduction capacity is proportional to the increase in the concentration of the compounds (Fig. 5). These results agree well with (Haddoudi and al 2014). At the concentration 1 mg/ml anthocyanins show a maximum optical density of 1.389 nm, followed by c-glycosides and total polyphenols with optical densities of 0.752 nm, 0.496 nm respectively. Aglycones show low reducing power with absorbance of 0.114 nm. All plant compounds show antioxidant activities significantly lower than the reference product (ascorbic acid) with absorbance of 3.011 nm (Fig. 5 and 6).

Anthocyanins and c-glycosides have the best scavenging powers towards free radicals (DPPH) (with IC50= 0.55 and 0.97 mg/ml respectively) and also the best Fe3+ reducing capacities (anthocyanins have a maximum optical density of 1.389 nm, followed by c-glycosides whose value is 0.752 nm). This probably indicates the presence in our compounds of powerful antioxidant molecules that can intervene by two types of reaction mechanisms (FRAP test and DPPH test). These data are in line with many studies that have evaluated the reducing effect of ferrous ions from extracts of various plants. The study conducted by Rubió and al (2013) shows that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity.) Bentabet and al (2014) indicate that there is a direct

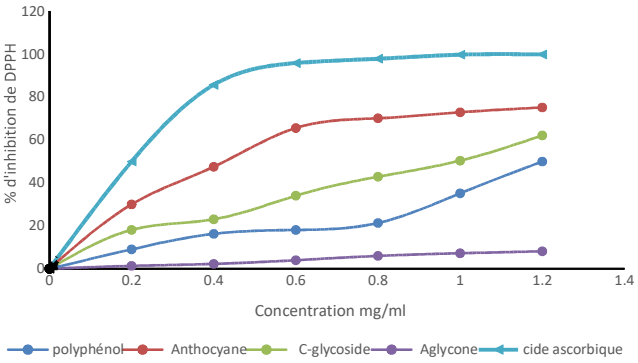


Fig. 3. DPPH uptake as a function of compound concentrations in *Marrubium vulgare* L.

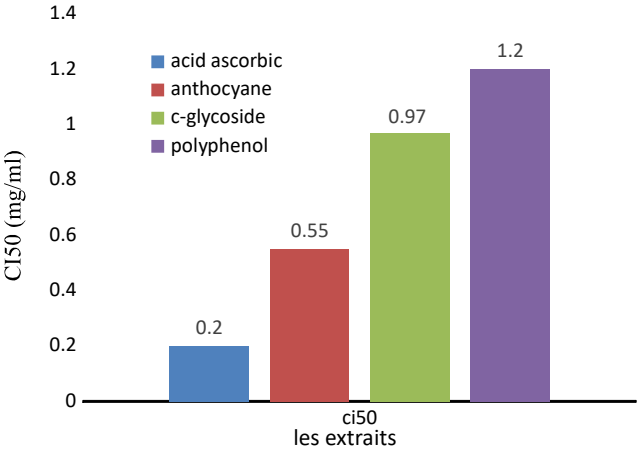


Fig. 4. Comparison of the IC50 of the different compounds

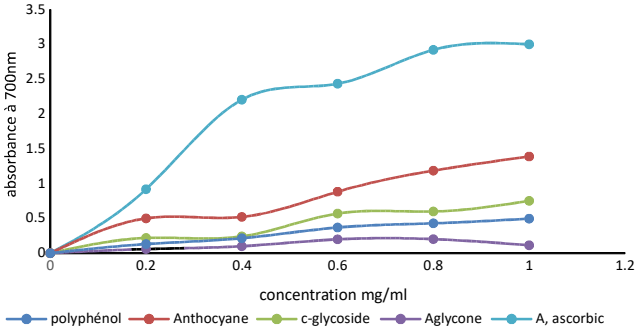


Fig. 5. Iron reducing power of the different compounds and ascorbic acid

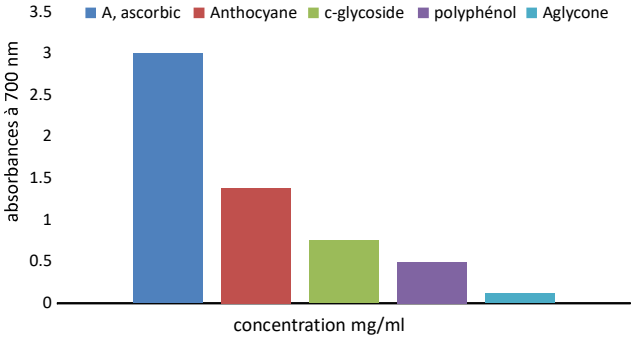


Fig. 6. Iron reducer of the different compounds and ascorbic acid at concentrations 1mg/ml

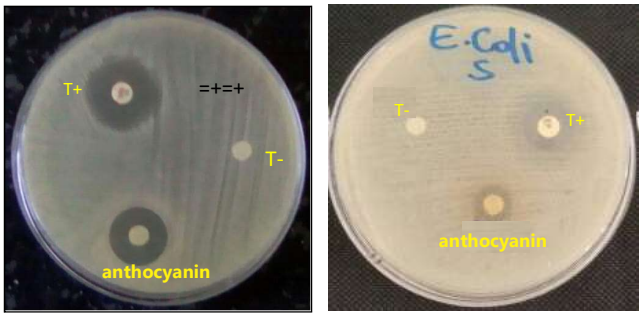
correlation between the antioxidant activities and the reducing power of the components of some plants.

Disc diffusion method (Antibiogram): The results of antibacterial activity tested on the two bacterial strains: *E. coli* and *S. aureus* are given in Figures 7 and 8, 9, 10.

The anthocyanins are the most active of all the other compounds (Fig. 7, 8, 9, 10, 11). This compound recorded the largest zones of inhibition against *Staphylococcus aureus* and *Escherichia coli* strains (16.5 mm and 13.50 mm respectively). The *S. aureus* strain was less sensitive to total polyphenols and c-glycosides (9 mm and 8.5 mm respectively). Total polyphenols and c-glycosides showed an interesting inhibitory activity towards the *Escherichia coli* strain with a diameter of 10 mm. Both strains are highly resistant to aglycones (6 mm).This inhibition activity is slightly lower than that of the reference antibiotic: Gentamicin. The negative control did not exert any inhibitory activity, the colonies develop normally in its presence and the anthocyanins marked a strong sensitivity towards the two strains.

Determination of the minimum inhibitory concentration(MIC): The bacterial growth decreases with the increase of the concentration of the compounds. The lower the MIC, the better the antibacterial activity of the compounds (Table 2). The MICs show that the *Escherichia Coli* strain is sensitive at both concentrations 1/4 and 1/2 for anthocyanins (MIC= 1/8) and also sensitive at both concentrations 1/4 and 1/8 for c-glycosides (MIC=1/32). For total polyphenols the MIC is undetermined.

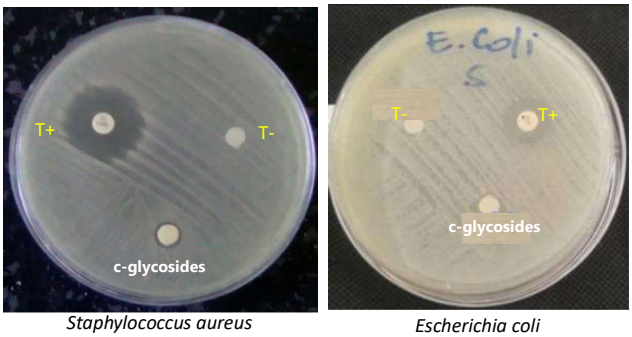
The *Staphylococcus aureus* strain seems very sensitive to concentrations 1/2 and 1/4 for anthocyanins (MIC =1/16) and moderately sensitive to concentration 1/2 for c-glycosides (MIC=1/8). *Staphylococcus aureus* is very resistant to total polyphenols (Fig. 12, 13, and 14). These results reveal that the Gram+ *Staphylococcus aureus* bacterium was more sensitive to anthocyanins and c-



Staphylococcus aureus *Escherichia coli*

(T+: Gentamicin; T- : Diethyl ether)

Fig. 7. Zones of anthocyanin inhibition



Staphylococcus aureus *Escherichia coli*

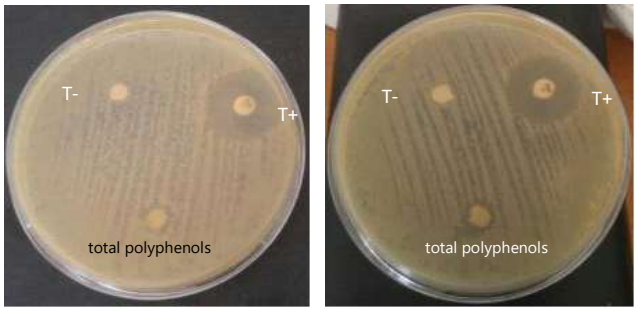
T+: Gentamicin positive control (.antibiotic) T- : n-butanol

Fig. 8. Zones of inhibition of c-glycosides

Staphylococcus aureus. *Escherichia coli.*

T+: Gentamicin positive control (.antibiotic) T- : negative control (methanol)

Fig. 9. Zones of inhibition of aglycones



Staphylococcus aureus. *Escherichia coli*

T+: Gentamicin positive control (.antibiotic T- : methanol)

Fig. 10. Zones of inhibition of total polyphenols

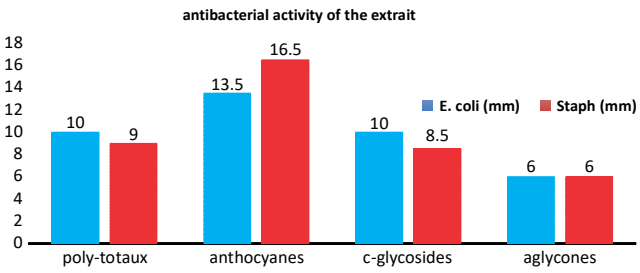
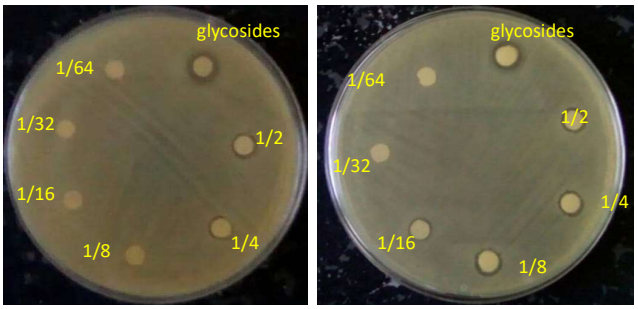


Fig. 11. Diameters of inhibition of the different compounds tested on the two bacteria

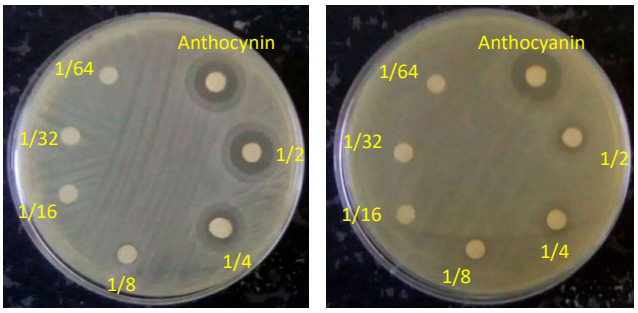
Table 2. Diameter of the inhibition zone (mm)

			Total polyphenols				
Bacteria total polyphenols	1/2	1/4	1/8	1/16	1/32	1/64	
S. aureus	00±00	00±00	00±00	00±00	00±00	00±00	
E. coli	9±0.741	9±0.741	10±0.741	9±0.741	9.5±0.741	8±0.741	
Anthocyanins							
Bacteria	anthocyanes	1/2	1/4	1/8	1/16	1/32	1/64
S. aureus	17.5±4,84	17±4.84	15.5±4.84	7.5±4.84	00±00	00±00	00±00
E. coli	15±3.90	8.5±3.90	8±3.902	00±00	00±00	00±00	00±00
C- glycosides							
Bacteria	c-glycosides	1/2	¼	1/8	1/16	1/32	1/64
S. aureus	13.5±1.75	11±1.75	10±1.75	00±00	00±00	00±00	00±00
E. coli	12±1.25	10±1.25	10±1.25	10.5±1,25	8.5±1.25	00±00	00±00



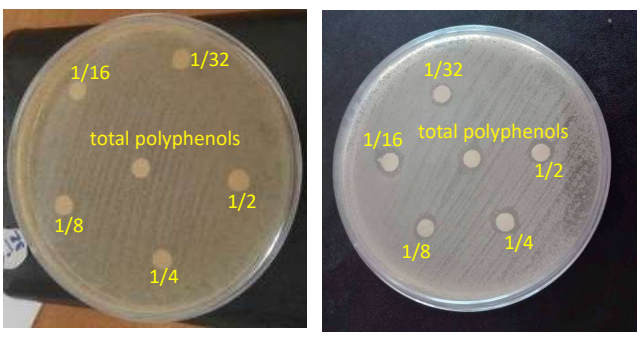
Staphylococcus aureus *Escherichia coli*

Fig.12. MIC of c-glycoside



Staphylococcus aureus *Escherichia coli*

Fig. 13. MIC of Anthocyanin



Staphylococcus aureus *Escherichia coli*

Fig.14. MIC of total polyphenols

glycosides than the Gram- *Escherichia coli* bacterium. This is consistent with recent studies on Lamiaceae that showed marked antibacterial activities against both types of bacteria with high inhibition against Gram (+) (Vijayabaskar 2012,Krichen 2015 and Bemmansour and al 2021). The resistance of Gram (-) bacteria could be explained by the presence of outer membranes that surround the cell wall and limit the diffusion of hydrophobic compounds through the covering lipopolysaccharides (David and Sudarsanam 2013).

CONCLUSION

The phytochemical study of the methanolic extract of the leaves and flowers of *Marrubium vulgare L* species revealed the presence of total polyphenols, flavonoids mainly anthocyanins, C-glycosides and aglycones. The antioxidant activity by DPPH and FRAP indicate that anthocyanins have the highest anti-radical activity followed by C-glycosides. The antioxidant activity of *Marrubium vulgare* L extract highlighted in this study could justify the traditional uses of this plant to develop new bioactive compounds. For the antibacterial activity, the most active compounds against *Staphylococcus aureus* and *Escherichica coli* are anthocyanins and c-glycosides. Therefore, these results remain promising, and could serve as a basis for further studies to confirm the antimicrobial efficacy of these natural products and to propose their use as antimicrobial agents, compensating for the side effects of antibiotics and increased bacterial resistances

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