EVALUATION OF THE PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY OF THE HYDROMETHANOLIC EXTRACT OF *Cynomorium coccineum* COLLECTED IN THE ALGERIAN SAHARA

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Abstract. The use of medicinal plants is well developed in the wilaya of Adrar, and this modest work is only a small attempt to better understand these medicinal plants used traditionally. In this study, we are interested in evaluating the antibacterial activity of the medicinal plant *Cynomorium coccineum*, characterized by being a medicinal plant, and a spontaneous species. The antibacterial study was carried out against six bacterial strains (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis)*. In fact, the extract of *Cynomorium coccineum* exerted a good inhibitory effect against four strains out of the six strains studied (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli*). and For the *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* strains, the MIC obtained is equal to 25 mg/mL. For this study, the minimum inhibitory concentration (MIC= 25 mg/mL) values obtained enabled us to confirm the results of the diffusion tests on disks initially carried out.

Key words: medicinal plants; *Cynomorium coccineum*; antibacterial; the minimum inhibitory concentration.

INTRODUCTION

The interest of medicinal plants lies in all the natural substances derived from these plants and which are used in the field of phytotherapy. The secondary metabolites are the active principles of medicinal plants and which are at the origin of these therapeutic virtues. They are represented by three main groups (terpenes, alkaloids and phenolic compounds). We have recently witnessed the rediscovery of natural substances as a potential reservoir of innovative therapeutic solutions for human health, with the prospect of integrating and sometimes replacing conventional drugs. *Cynomorium coccineum* is a well-known holoparasitic plant in ethnopharmacology, although its current use as a curative remedy is reported only in certain ethnic groups in North Africa and the Arabian Peninsula.

Cynomorium coccineum is present in almost all of the Mediterranean basin. Only recently has research begun to confirm some of its traditional uses. To highlight previously unknown biological activities, many recent scientific discoveries have focused on the phytochemistry of the plant, such as some of its antioxidant and biological activities (antimicrobial, anticancer, pro-erectile and anti-tyrosinase) as well *in vivo* than *in vitro*. Some of them may be promising from the point of view of food and cosmetic formulations [20].

The study conducted by Vascellari *et al*. in 2021 [28], on the chemical and biological properties of *C. coccineum*, evaluating the potential antiviral and antiproliferative activity of the methanolic extract, revealed an interesting antiproliferative activity against cell lines derived from human leukemias.

A study by Rosa *et al*. in 2015 [25], examine the anticancer properties of fixed oil, obtained from *Cynomorium coccineum*, on B16F10 melanoma and the viability and lipid profile of Caco-2 colon cancer cells, highlighted the importance of this oil rich in essential fatty acids. It has shown the existence of a significant inhibitory effect on melanoma and colon cancer cells. Incubation (24h) with concentrations of non-toxic oil (25 and 50 µg/mL) induced in the two cancer cell lines a significant accumulation of fatty acids and an increase in the level of eicosapentaenoic acid, having an activity anticancer. All these results show the importance of *Cynomorium coccineum* extracts and the promising potential in cancer prevention and other pharmaceutical applications [25].

Another author cites *Cynomorium coccineum* as a traditional remedy for wound healing [10]. The antimicrobial activity of *Cynomorium coccineum* extracts should be widely studied, as it would also be a favorable characteristic in nutraceutical or cosmetic formulations [20, 31].

Our work is a modest contribution to the various studies on the extracts of *Cynomorium coccineum*, and aims to highlight the importance of this plant and thus deepen the knowledge and possible applications of this plant.

Our objective is to highlight the antibacterial effect of the hydromethanolic extract of *Cynomorium coccineum*, collected at the level of the wilaya of Adrar.

MATERIALS AND METHODS

Study zone

This study was carried out at the level of the wilaya of Adrar, located in the central Sahara, which is classified by E. De Martonne, as absolute desert [13]. The wilaya of Adrar is located in the south-west of Algeria. It covers an area of 427,971 km^2 and its population is made up of 431,270 inhabitants, it includes eleven (11) daïras and twenty-eight (28) communes [4]. It is divided into four geographical areas; the Gourara, the Touat, the Tidikelt, the Tanezrouft.

Plant material studied "*Cynomorium coccineum*"

Arabic name: Tartout, and the vernacular name in Adrar: Danoun. The collections were carried out during the month of January 2021. Once the plants were picked, they were washed, dried in the open air for about twenty days, at room temperature in a room and protected from light, and finally crushed to obtain a fine powder.

Phytochemical tests

We have adopted a protocol proposed by Bouchouka in 2016 [8]. The objective is to identify the secondary metabolites present in this plant. To check the presence or absence of flavonoids, we mixed 2 g of the powder with 40 mL of 1% (HCl), macerated for 24 hours.

A few drops of pure (NH_4OH) are added to 10 mL of the filtrate, if there is presence of flavonoids then a light yellow color appears in the upper part of the test tube. And to get an idea if there are Tannins in the plant, we infuse 2 g of the powder in 20 mL of water and after 5 minutes we filter.

We added 5 mL of the filtrate to 15 mL of distilled water and a few drops of 10% ($FeCl_3$) are added, if a blue or green color appears then tannins are present. For saponins, 2 g of powder should be infused in 20 mL of water and then 2.5 mL of filtrate is put in a test tube and 10 mL of distilled water is added.

The mixture is stirred for a few seconds and left to stand and after half an hour if the foam persists at least 1 cm, this indicates the presence of saponins. And finally, to check the presence of Anthocyanins, 2g of the powder (10%) is infused in 20 mL of water and a few drops of pure hydrochloric acid (HCl) are added and a color change is observed. After the first color change a few drops of ammonium hydroxide (NH_4OH) are added to this mixture, a second color change is observed which indicates the presence of anthocyanins (the pH change induces a color change).

Preparation of hydromethanolic extracts of *Cynomorium coccineum*

The objective is to extract the secondary metabolites contained in the plant, using methanol as a solvent, according to the method described by Abdallah in 2017 [1]. We take 10 g of the powder wich are macerated in 100 mL of a solvent of a mixture consisting of Methanol -water ($MeOH/H_2O$); (80/20) by volume.

The macerate is filtered after 3 days of incubation at 37°C and then evaporated at 45°C under reduced pressure, using a rotary evaporator.

The semi-solid crude extract obtained is dried at 50°C for two days, in order to obtain a dry crude extract. The dry extract is dissolved and taken up in a 100% pure solution of DMSO (di-methyl sulfoxide).

The hydromethanolic extract thus obtained represents the stock solution with a concentration of 500 mg/mL for carrying out the antibacterial screening.

Method of calculating the yields of the hydromethanolic extract

The percentage yield (%) is the ratio between the mass of dry extract obtained and the mass of the powder of the plant used (after drying and grinding). calculated by the following formula:

Yield % = (Weight of crude extract / Weight of dried and ground plant powder) × 100

Preparation of the inoculums

The antibacterial tests are carried out on six bacterial strains (table 1).

The resuscitation of the strains was carried out using nutrient agar, incubated at 37°C for 24 hours. Then, a few colonies isolated from a pure culture, of each strain, are scraped, then injected into 10 mL of physiological water.

Whose opacity of this bacterial suspension must be equivalent to 0.5 MFU Mc Farland Units, corresponds approximately to a culture density of 1.5 x 10^8 cells/mL, i.e. an optical density (OD) equal to 0.08 at 0.10, read at the wavelength of 625 nm.

Evaluating the antibacterial activity of the extract of *Cynomorium coccineum* (Disc broadcast method)

This is the method used by Gherairia *et al.,* in 2019 [14]. It is based on measuring the diameter of the microbial growth inhibition zone around an antibacterial source deposited on the surface of a pre-tested Mueller-Hinton agar seeded.

The hydromethanolic extract of *Cynomorium coccineum* will be tested against six bacterial strains according to the "disk diffusion method", if the effect is positive, we move on to the study of the minimum inhibitory concentration (MIC) and bactericidal (CMB) in a solid medium.

Discs of Whatman filter paper are impregnated with 10µL of the hydromethanolic extract of *Cynomorium coccineum* and deposited on the medium inoculated with one of the six bacterial strains.

Three repetitions are carried out for each extract (03 discs of the same extract per box). The fourth disc is a negative control which is impregnated with pure DMSO solvent, the fifth disc is the positive control

Table 1. The bacterial strains tested and their origins

Bacterial strains	References	Gram
S1: Staphylococcus aureus Rosenbach	ATCC 25923	Positive
S2: Bacillus cereus Frankland & Frankland	ATCC 11778	Positive
S3: Bacillus subtilis (Ehrenberg) Cohn	ATCC 23857	Positive
S4: Escherichia coli T. Escherich	ATCC 25922	Negative
S5: Enterococcus faecalis Schleifer & Kilpper-Bälz	ATCC 29212	Positive
S6: Pseudomonas aeruginosa Migula	ATCC 27853	Negative

ATCC: American Type Culture Collection.

which is impregnated with an antibiotic "Streptomycin". The measurements of the zones of inhibition are carried out after 24 hours of incubation at 37°C.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined according to the method cited by Chouikh *et al.,* in 2015 [11].

The MIC corresponds to the smallest concentration causing inhibition of the growth of a bacterial strain. We prepared six volumes ranging from (0.3 mL) to (3 mL) which were adjusted to 10 mL, with Muller Hinton medium. Thus we obtained the following concentrations (15 mg/mL, 25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 125 mg/m, 150 mg/mL). Each mixture, medium extracted from Muller Hinton, is poured into a Petrie dish.

After solidification, the bacterial strains are seeded using swabs. Observation and comparison of results begin after 24 hours of incubation at 37°C [11].

Determination of Minimum Bactericidal Concentration (MBC)

It is defined as the lowest concentration capable of killing 99.9% of bacteria. The MBC assay was carried out after subculturing on Mueller Hinton Agar and incubated at 37°C for 24 hours using swabs. Transplantation retains only the bacterial strains that showed no bacterial growth in the MIC test.

Statistical Analysis

All data in this study were statistically analyzed using one-way ANOVA and Tukey post-hoc multiple comparison tests to analyse the zone of inhibition of antibacterial effect.

RESULTS

Results of *Cynomorium coccineum* phytochemical tests

Phytochemical tests have shown the presence of certain types of secondary metabolites. These results gave us an idea of the chemical composition of *Cynomorium coccineum*, see figure 1, and table 2.

Yields of hydromethanolic extract of *Cynomorium coccineum*

The result shows that the yield of secondary metabolites of *Cynomorium coccineum* is slightly high. Our *Cynomorium coccineum* extract gave a yield equal to 1.825g (18.25%). This result is consistent with that obtained by Dhahir and Kredy in 2021 who obtained a yield of 1.0211g [12].

Yield % = (Weight of crude extract / Weight of plant powder) × 100 = (1.825/10)100 = 18.25%.

Antibacterial evaluation of *Cynomorium coccineum* extract by the disc Diffusion Method

This is the method described by Gherairia *et al.,* in 2019 [14]. it makes it possible to qualitatively assess the antibacterial activity *in vitro*. It highlights the sensitivity or not, of a bacterial strain to the extract studied. We tested the antibacterial effect of our extract against the following six bacterial strains:

Strain 1 (S1): *Staphylococcus aureus* Rosenbach.

Strain 2 (S2): *Bacillus cereus* Frankland & Frankland.

Strain 3 (S3): *Bacillus subtilis* Ehrenberg.

Strain 4 (S4): *Escherichia coli* T. Escherich.

Strain 5 (S5): *Enterococcus faecalis* Schleifer & Kilpper-Bälz.

Strain 6 (S6): *Pseudomonas aeruginosa* Migula.

The antibacterial activity is determined when a zone of inhibition of bacterial growth appears around the extract studied [9]. The measurement of the inhibition diameter is carried out with a simple ruler. The results represent the average of three replicates. The presence of a zone of inhibition demonstrates the existence of an obvious sensitivity of the strains studied with respect to the extract tested [2, 5], the sensitivity of a bacterial strain can be classified according to its diameter inhibition as follows:

-Not sensitive or resistant: diameter < 8mm

-Sensitive: diameter between 9 to 14mm

-Very sensitive: diameter between 15 to 19mm

-Extremely sensitive: diameter > 20mm

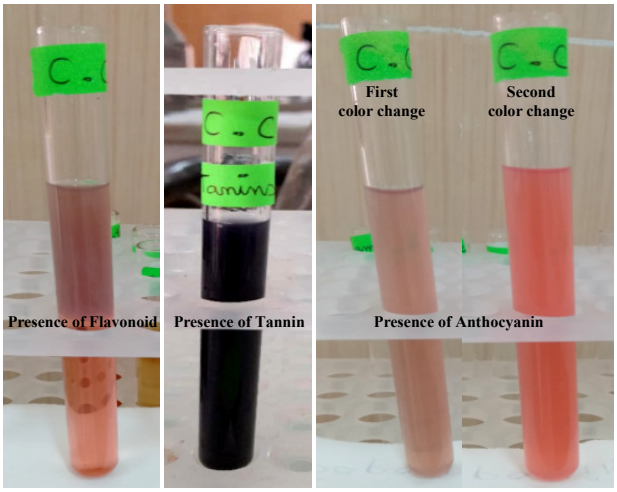

Figure 1. Highlighting the presence of Tannins, Flavonoids and Anthocyanins

Table 2. *Cynomorium coccineum* phytochemical test results

Plant tested	Flavonoids	Tannins	Saponins	Anthocyanins
Cynomorium coccineum	+ ++	+ + +	-	+++

+ + +: Very positive reaction; ++: Moderately positive reaction; +: Weakly positive reaction: -: Negative reaction.

The extract of *Cynomorium coccineum* was tested *against Staphylococcus aureus, Bacillus cereus, Bacillus subtilis* and *Escherichia coli*, whose diameter of the zones of inhibition varies from 11mm to 12mm.

The *Enterococcus faecalis* and *Pseudomonas aeruginosa* strains are not very sensitive to the action of the extract, and the diameters of the zones of inhibition vary from 07mm to 08mm. Pure DMSO used as a control showed no zone of inhibition (Fig. 2).

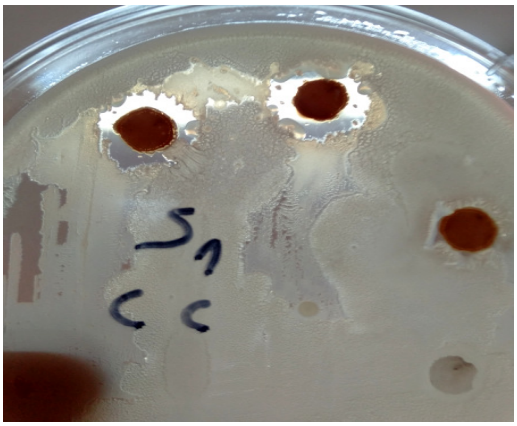

Figure 2. Zones of inhibition of *Cynomorium coccineum* extract against bacterial strains S1: *Staphylococcus aureus.*

Our result is similar to that obtained by Dhahir and Kredy in 2021 [12] in which different concentrations of *Cynomorium coccineum* demonstrated efficacy against the following different bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus saprophyticus, Streptococcus agalactiae and Klebsiella pneumonia*). Our result is consistent with that obtained by Yusuf *et al.,* in 2018 [30], whose plant extracts had an inhibitory effect on several pathogenic microorganisms such as bacteria, fungi and yeasts due to the presence of several active compounds such as tannins, flavonoids and polyphenols.

The antibacterial activity that we obtained is very probably due to the flavonoids and anthocyanins and tannins present in good proportion in our extract of *Cynomorium coccineum* this is exactly what several authors confirm to us, such as Bhattacharyya *et al.,* in 2011[7] and Harborne in 1972 [16], who found a high percentage of phenolic compounds in their extracts of *Cynomorium coccineum*.

They believe that this high level may be the source of their antibacterial activity, and whose phenolic compounds present in plant extracts cause the destruction of the membranes and cell walls of these microscopic organisms [7, 15]. Our results also confirm those obtained by Zucca *et al.,* in 2019 [31], who tested the aqueous extract by the disk diffusion method against several Gram-positive and negative bacterial strains [21], they observed that their extract inhibited the growth of all Gram-positive bacteria tested, including the MRSA clinical isolate *Staphylococcus aureus* (methicillin resistant).

Our result was consistent with that obtained by Marin *et al.,* in 2015 [22], who further reported that anthocyanins interfere with the permeability of bacterial membranes [22]. Zucca *et al.,* in 2019 [31], reported that the antibacterial activity was not impaired by the nearly anthocyanin-free extract obtained from the peeled plant [31]. This suggests that the antibacterial activity of *Cynomorium coccineum* could also be due to other phytochemicals such as gallic acid, which is the main component of this extract, this acid and its derivatives (gallates) are said to have antimicrobial activity, probably due to their pro-oxidant effect [18, 21]. In our statistical analysis we represented the averages of the zone of inhibition measured by letters a and b.

The inhibition means of the bacterial strains tested which have the same letter are not significantly different at P = 0.05 (Fig. 3).

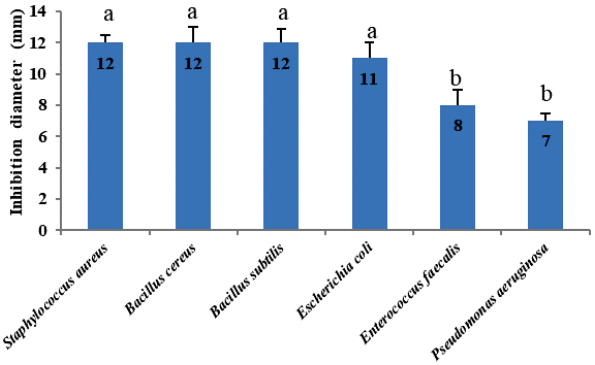

Figure 3. Antibacterial effect of *Cynomorium coccineum* crude extract. Data are expressed as mean ± standard deviation (n = 3). Means of inhibition with the same letter are not significantly different at *P* = 0.05.

Determination of the Minimum Inhibitory and Bactericidal Concentration of extract of *Cynomorium coccineum*

To determine the minimum inhibitory and bactericidal concentrations (MIC and MBC), we prepared six volumes ranging from 0.3 mL to 3 mL, the volumes prepared correspond to the following concentrations 15 mg/mL, 25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 125 mg/mL, 150 mg/mL. We tried to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). The strains retained for this work are only those sensitive to the extract of *Cynomorium coccineum*, whose inhibition diameter varies from 10 to 12mm, namely (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli)*. The MIC and MBC values were obtained according to the

method of Berche *et al.,* in 1991 [6]. The results are presented in figure 4, figure 5 and table 3.

*** Minimum Inhibitory Concentration (MIC) of *Cynomorium coccineum***

For the *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* strains, the MIC obtained is equal to 25 mg/mL. For the strain *Bacillus subtilis* the MIC has not been determined (table 3 and figure 4).

Minimum Bactericidal Concentration (MBC) of *Cynomorium coccineum

For *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis* and *Escherichia coli*, the MBC was not determined (Table 3 and Fig. 5).

DISCUSSIONS

Phytochemical tests, of *Cynomorium coccineum*, revealed the presence of flavonoids and tannins in very good proportion as well as anthocyanins, but there is an absence of saponins. The results of the antimicrobial evaluation of *Cynomorium coccineum* showed antibacterial activity for the strains *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*, *Escherichia coli*. S5 and S6 strains were not sensitive to the hydromethanolic extract of *Cynomorium coccineum*. We also found that the antibacterial activity of *Cynomorium coccineum* extract is relatively high, it is probably induced by some phytochemicals present in *Cynomorium coccineum*. The values of the minimal inhibitory concentrations of *Staphylococcus aureus, Bacillus cereus, Escherichia coli* are equal to 25 mg/mL. No minimum bactericidal concentration value was observed. The minimum inhibitory concentration (MIC) values obtained enabled us to confirm the results of the diffusion tests on disks initially carried out. We know that *Staphylococcus aureus* (S1) is the most pathogenic species, of the genus *Staphylococcus*, it is responsible for food poisoning, localized suppurative infections.

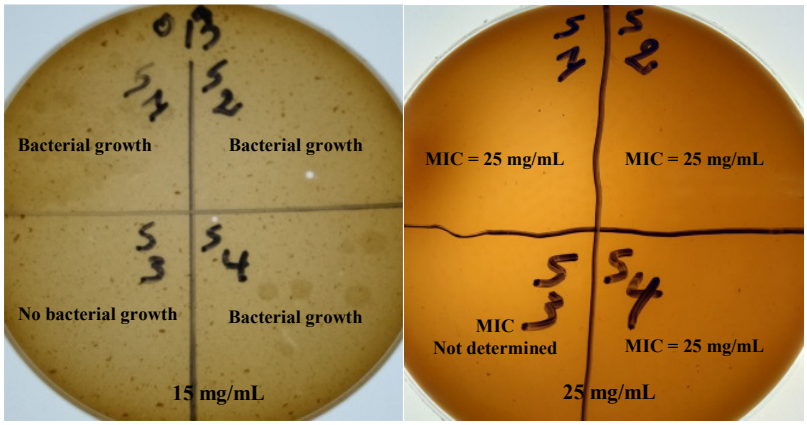

Figure 4. The minimum inhibitory concentration (MIC) of the *Cynomorium coccineum* extract against the bacterial strain S1: *Staphylococcus aureus*, S2: *Bacillus cereus*, S4: *Escherichia coli*. And bacterial growth of the bacterial strain S3: *Bacillus subtilis* under the effect of the extract of *Cynomorium coccineum* at a concentration of 15 mg/mL.

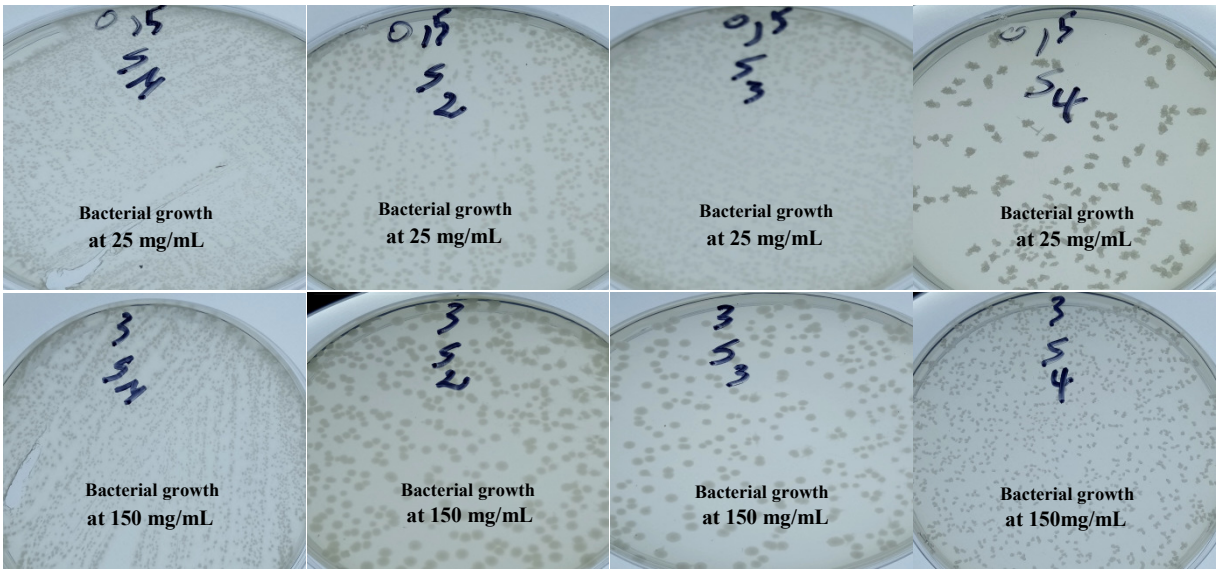

Figure 5. No minimum bactericidal concentration (MBC) of *Cynomorium coccineum* was determined and bacterial growth was observed in bacterial strains: (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis* and *Escherichia coli*) from the concentration of 25 mg/mL to that of 150 mg/mL.

Table 3. The MIC and The MBC of the *Cynomorium coccineum* extract

Extracts from the plant of	S1 : *Staphylococcus aureus*	S2 : *Bacillus cereus*	S3 : *Bacillus subtilis*	S4 : *Escherichia coli*
Cynomorium coccineum	MIC = 25 mg/mL and MBC Not determined	MIC =25 mg/mL and MBC Not determined	MIC and MBC Not determined	MIC =25 mg/mL and MBC Not determined

Certain strains of *Escherichia coli* (S4) are responsible for various pathologies in humans such as diarrhea and gastroenteritis. Phytochemical tests performed on *Cynomorium coccineum* powder revealed the presence of flavonoids, tannins and anthocyanins, and flavonoids are considered antimicrobial agents, against many bacterial strains such as (*Staphylococcus aureus, Escherichia coli, Enterococcus feacalis, Enterobacter cloaceae, Heliotropium sinuatum, Proteus mirabilis*) [3, 16, 23, 27]. In the light of all these data, it can be assumed that the secondary metabolites responsible for the antibacterial effect are probably the flavonoids and/or the tannins and/or the anthocyanins present in the medicinal plant *Cynomorium coccineum* L. Drug resistance is not a new problem, indeed after the introduction of penicillin, staphylococci developed resistance to many antibiotics. This phenomenon of antibiotic resistance used to exist only in hospitals, but is now observed in the community. Bacteria such as *Staphylococcus* have emerged with resistance to six and more different antibiotics [26]. Our hydromethanolic extract of *Cynomorium coccineum* was active against 75% of the total gram-positive bacteria and 25% of the gram-negative bacteria we studied. Similar results were also demonstrated by Babayi *et al.* (2004) [3], whose Gram-positive bacteria were more sensitive than Gram-negative bacteria. Indeed our extract inhibited the bacterial growth of three Gram-positive strains (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*) and one Gram-negative strain (*Escherichia coli*). This is in agreement with a number of previous studies that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria [19, 24]. The active compounds present such as tannins, phenols and flavonoids have a certain antibacterial role in addition to the synergistic influence of these active chemical compounds between them, which together can contribute to this antimicrobial activity [17].

The difference in antibacterial effects of the extract observed between Gram-positive and Gram-negative bacteria is of course mainly due to the differences in the composition of the cell wall of these bacteria. Positive bacteria have only one layer in their cells, while Gram-negative bacteria have a multi-layered structure [29].

Cynomorium coccineum has been known for many centuries, it has been appreciated by European and especially Arab traditional medicine, which has used *Cynomorium coccineum* for the preparation of remedies against hemorrhages, diarrhea, dysentery, and disorders of the apparatus reproductive. We tried through antimicrobial tests, to highlight the importance that this species could have, in the search for new remedies against the many existing human pathologies in Algeria. Phytochemical tests performed on *Cynomorium coccineum* powder revealed the presence of tannins and flavonoids and anthocyanins. The modest results of our antimicrobial study revealed that *Cynomorium coccineum* is a plant of interest, indeed the antibacterial activity against the six bacterial strains tested, had a notable inhibitory effect. For this study, the minimum inhibitory concentration (MIC) values obtained enabled us to confirm the results of the diffusion tests on disks initially carried out. The results obtained are very encouraging and promising, in terms of antibacterial activity, the extract of *Cynomorium coccineum* exerted a good inhibitory effect against the four strains (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli*). Our hypothesis is that the secondary metabolites responsible for the antibacterial effect are probably flavonoids and/or tannins and/or antocyanins from *Cynomorium coccineum*. We can, to verify this hypothesis, consider later, other studies in vitro, and with other types of extracts, and even their fractions, as well as the evaluation of all their biological activities, to confirm or not the results, which we got. Our final result corroborates other similar results obtained by Zucca *et al.,* in 2019 [31]. Indeed our extract inhibited the bacterial growth of three Gram-positive strains (*Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*) and a Gram-positive strain -negative (*Escherichia coli*). It should be noted that our extract had a good antibacterial effect against the Gram-positive *Staphylococcus aureus* (S1) bacterial strain, known for its resistance developed in the hospital environment (methicillin resistance). We consider that this last result is important and encouraging to explore other fields of research around this very promising plant. Therefore, doing additional work is then desirable and even necessary. It is necessary to underline the importance of being able to extend and promote other multidisciplinary studies on the medicinal plants of the wilaya of Adrar.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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