

Study of the antioxidant and bacterial activity of the flavonic glycosides existing in the plant "Cynodon Dactylon (L) Pers" by the free radical DPPH and the reduction of iron

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Abstract

The phytotherapy use of *Cynodon Dactylon* "L" Pers plant in traditional medicine has led us to work on the evaluation of the heteroside extracts of this plant, as well as aiding in the performance of phytochemical screening and the assessment of this plant's antioxidant activity using the "FRAP" method for reducing iron and scavenging DPPH radicals. Furthermore, research on the flavonoid components found in the two plant extracts' antibacterial properties is needed.

The antioxidant properties were demonstrated by identifying the effective inhibition concentration IC₅₀ compared by the antioxidant effect of quercetin. The results mentioned that the extract of Ethyl Acetate has a higher activity than that of the Butanol extract "Ethyl Acetate extract IC₅₀ $\approx$ 0.014 mg/ml, butanol extract IC₅₀ $\approx$ 0.019 mg/ml". Also, the reducing power at the concentration of 2.5 mg/ml of ethyl acetate extract of *Cynodon Dactylon* was much higher (Ab=1.376 nm) compared to the Butanol extract (Ab= 1.297nm).

On the other hand, the butanol extract exerted weak activity against the referential bacterial strains. However, the ethyl acetate extract of flavonoids exhibited moderately positive activity on only two strains of "*Enterococcus faecalis* and *Streptococcus pyogenes*".

Keywords: Cynodon dactylon; glycosides; antioxidant activity; the radical DPPH; FRAP method

1. Introduction :

Polyphenols are natural compounds widely distributed in the plant kingdom which have a growing importance, due to their beneficial effects on health ¹⁶. Polyphenols and especially flavonoids are powerful antioxidants capable of inhibiting the formation of free radicals and opposing the oxidation of macromolecules ⁴². Indeed, flavonoids are effective scavengers of the most pro-oxidant free radicals, particularly involved in lipid peroxidation, since they prevent it like α -tocopherol. They formed intermediate radical species that are not very reactive ^{17,28}. Flavonoids are powerful inhibitors of oxidation of LDL (Low Density Lipoprotein) ¹⁷.

Due to their low redox potential, flavonols (Fl-OH) can thermodynamically reduce free radicals (R $\cdot$), such as superoxide, peroxy, alkoxy, and hydroxyl, by hydrogen transfer; the resulting radical, flavonoxyl (Fl-O $\cdot$), can then react with another radical to produce a stable quinone structure.

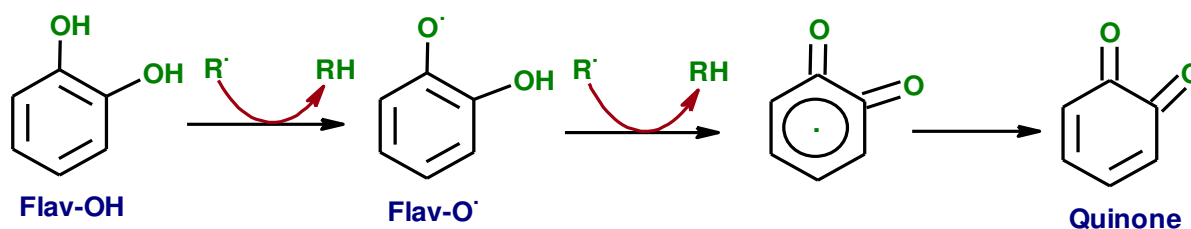
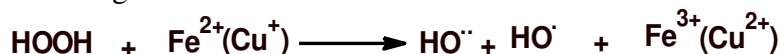


Fig 1 : Trapping of ROS (Reactive oxygen species) (R.) by flavonoids.

Flavonoids are also considered as an effective chelators of metal ions ^{29,42}, such as iron (Fe²⁺), and copper (Cu⁺) ions which are essential for certain physiological functions, but they are also responsible for the production of hydroxyl radical by the reduction of hydrogen peroxide according to the following reaction:



Quercetin is the most active of the flavonoids studied. Figure 2 summarized the essential sites for metal ion chelation: (a) a catechol ring on the B ring, (b) the 3-hydroxyl and 4-oxo groups on the C ring, and (c) the 4-oxo groups and 5-hydroxyl between rings A and C ⁴².

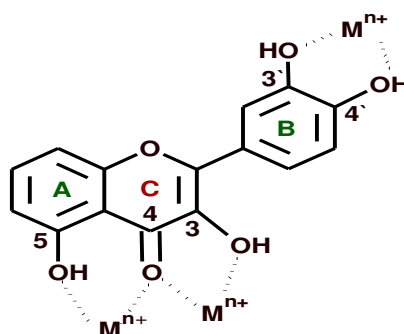


Fig 2: Flavonoids and their sites proposed for chelation metal ions (Mn+)
(From Van Acker S., and al 1995)

Furthermore, the flavonoids were able to act at the level of the synthesis of viral proteins thus allowing a good protection of mice vis-à-vis a viral infection following a daily administration of 3-O-methylquercetin at 20 mg/kg for 9 days ³⁴.

Previous studies highlighted an impact of flavonoids on the HIV retrovirus responsible for acquired immunodeficiency syndrome (AIDS).

They demonstrated that flavonoids are good reverse transcriptase inhibitors. However, their impact seems stronger on the DNA and RNA polymerase of the host cell than on the viral reverse transcriptase. ^{25,39,18}. Moreover, various studies have provided evidence of the bactericidal effect of different flavanones on the DNA gyrase from a *Staphylococcus aureus* bacterium. ²⁴.

Naturally, flavonoids are very often found in the form of glycosides, where there are two types of heteroside flavonoids ⁹.

- O-heteroside
- C-heteroside

The bond between genin and ose can generally be performed with the hydroxyls in position “7” of flavones and hydroxyls in position “3” of flavanols which are involved in the case of O-glycosil.

C-heterosides: more than 300 are known, the bond was established between the anomeric carbon of sugar and carbon in position 6 or 8 of the genin ¹⁰.

Previous research were carried out in extraction, identification, and quantification of these compounds from different sources such as agricultural and horticultural crops or medicinal plants such as *Cynodon dactylon* "L" Pers.

In addition, *Cynodon dactylon* "L" Pers (quackgrass) known as a perennial plant found in Asia, Europe, North America, and North Africa. This plant was well known to gardeners who fear its spread both on the surface and underground. Although, Quackgrass, which gardeners frequently refer to as a weed because of its destructive effects on the soil, but possesses medicinal qualities that are very beneficial to human body ¹⁴.

Because of the potassium salts found in its stem and rhizome, **Quack grass** has a long history of being used by Greek doctors as a diuretic and kidney stone remover despite its unfavorable reputation, it is also used for its febrifuge effects. Quack grass has been reported as an emollient, fighting urinary problems such as urethritis and cystitis. It protects the urinary tract. In decoction, it relieves inflammation of the prostate. Furthermore, Quack grass prevents the recurrence of renal colic served as an infusion, it fights against cellulite ¹⁴. Also, *C.dactylon* leaf extract has been reported to have antidiabetic, ^{30,36} antioxidant, hypolipemia, ^{15,29,34} healing of minor injuries ²⁶, and hepatic antioxidant properties ³².

Additionally, we were interested in researching the antioxidant properties of the plant *Cynodon dactylon* "L" Pers, which is grown on one of the farms in the Ouargla region, three kilometers to the northeast of the city of Ouargla, extends over an area of 360 m², it includes 60 palm trees plus palm tree shoots. It is a perennial grass with a long creeping rhizome on the surface of the soil about 20 m long, it is much branched bearing many erect leaves with long sheaths surrounding the stem and a flattened blade 2.5-20cm long ⁶.

The inflorescence composed of 2 to 6 digitate ears, straight, purple or green with villous rachis at least at the base, arranged in two tight rows, in ears appearing toothed 50 mm long at most, the flowering period extends from February to November ^{3,6}.

The current study was devoted to deducing the antioxidant activity of the two extracts Ethyl acetate and Butanol according based on the method of scavenging of free radical DPPH and that of the reduction of iron FRAP.

2. Materials and Methods :

2.1. Extraction and determination of flavonic glycosides :

The extraction method used was performed according to Foungebe et al. (1976). This method allowed the extraction of polyphenolic compounds, in particular flavonoids.

The ethanoic extracts were obtained by maceration and agitation of plant material for 24 hours in an ethanol/water mixture (80/20: V/V). The solvent/plant material ratio was 10/1 (ml/g) ¹⁹.

The extract was concentrated using rota-steam at 40°C under reduced pressure. The residue was taken up with boiling water (600ml) and the aqueous phase was degreased with diethyl ether (3×200 ml) then extracted with ethyl acetate (3×200 ml) under evaporation of organic phase at dry of 40° C. The residue was then removed using 5 ml of methanol.

Moreover, the aqueous phase was extracted with butanol (3200 ml), the butanol phase was evaporated to dryness at 40°, and the residue was recovered with 10 ml of methanol.

Mono heterosides, aglycones, and occasionally diheterosides are present in the ethyl acetate extract while poly heterosides and C-glycosides are present in the butanol extract ²⁹.

2.2. Calculation of yields in dry extracts:

The following ratio can be used to calculate the plant's yield in dry heteroside extracts (butanol and ethyl acetate) [7].

$$Y_t(\%) = \frac{W_1 - W_2}{W_3} \times 100$$

Y_t: Total yield.

W₁ : Flask weight after evaporation. .

W₂ : Weight of empty flask.

W₃ : Weight of the starting plant material.

2.3. Activité anti oxydante des flavonoid heterosides :

2.3.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay:

- **Principle:**

The 2,2-diphenyl-1-picrylhydrazyl (α,α -diphenyl- β -picrylhydrazyl) (DPPH) was considered as one of the first free radicals used to study the antioxidant structure-activity relationship of phenolic compounds ^{1,2}. It has an unpaired electron on an atom of the nitrogen bridge (**Fig.1**). DPPH is a purple free radical that turns yellow when reduced by an H atom donor.

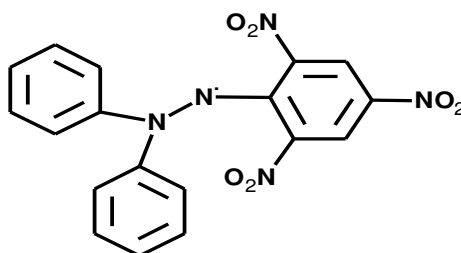


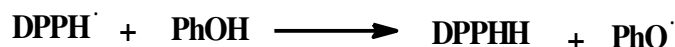
Fig 3 : The free radical DPPH• (2,2 DiPhenyl-1-Picryl-Hydrazyl)

The scavenging of free radicals by antioxidants was dependent on two types of mechanisms:

a/ The release of the hydrogen atom from the hydroxyl group (rapid kinetics of certain acids and phenolic derivatives).

b/ The release of an electron (slow kinetics of glycolysis derivatives and anthocyanins)^{11,21}

In the case of phenolic compounds (Φ -OH), the main mechanism of action is the trapping of free radicals by the transfer of the H atom on the DPPH• then transformed into a stable molecule DPPHH ^{17,29}.



- **Mode opératoire :**

A range of concentrations (0-200 $\mu\text{g/mL}$) of plant extract or quercetin (reference antioxidant) was prepared in methanol. A volume of 2.5 mL of this solution was mixed with 2.5 mL of DPPH (100 μM) also prepared in methanol. After homogenization, the mixture was incubated at room temperature (25°C) protected from light. After 30 minutes of incubation, the absorbance was recorded at 517 nm in the presence of blank which contains only methanol. The percentage inhibition of DPPH radical (I%) was calculated according to the following equation:

$$I\% = (1 - (\text{DO essai} / \text{DO blanc})) \times 100$$

I% : Radical inhibition percentage

OD : Optical density.

The graph showing the percentage of inhibition of DPPH as a function of the concentrations of extracts and Quercetin was used to determine the IC₅₀, which was the concentration of plant extract or Quercetin responsible for 50% inhibition of DPPH radicals.

2.3.2. Ferric reducing antioxidant power (FRAP) test:

- **Principle :**

The reducing power of an extract was associated with its antioxidant power. This technique was developed to measure the ability of the tested extracts in order to reduce the ferric iron (Fe^{3+}) present in $\text{K}_3\text{Fe}(\text{CN})_6$ complex to ferrous iron (Fe^{2+}). Indeed, Fe^{3+} participated in the formation of hydroxyl radical by the Fenton reaction. The absorbance of the reaction medium was determined at 700 nm ²⁷. The results exhibited an increase in absorbance corresponded to an increase in the reducing power of the extracts tested ¹².

- **Operating mode :**

The reducing power of iron (Fe^{3+}) in the extracts was determined according to the method described by Oyaizu 1986. One milliliter of the extract at different concentrations from 0.007 to 2.5mg/ml was mixed with 2.5ml of 0.2 M phosphate buffer solution (pH 6.6) and 2.5ml of 1% solution of potassium ferricyanide $\text{K}_3\text{Fe}(\text{CN})_6$. The mixture was incubated in water bath at 50°C for 20 min, then 2.5ml of 10% of trichloroacetic acid was added in order to stop the reaction and the tubes were centrifuged at 3000 rpm for 10 min. An aliquot (2.5ml) of supernatant was combined with 2.5ml of distilled water and 0.5ml of an aqueous solution of FeCl_3 at 0.1%. The absorbance was recorded at 700 nm against a similarly prepared blank, replacing the

extract with distilled water which makes it possible to calibrate the device (UV-VIS spectrophotometer). The positive control was represented by a solution of a standard antioxidant; quercetin whose absorbance was measured under the same conditions as the samples. An increase in absorbance corresponded to an increase in the reducing power of the extracts tested was recorded ³⁷.

2.4. Antibacterial activity :

Recent epidemiological research revealed that the extremely prevalent flavonoids found in food and medicinal plants have outstanding antibacterial and antioxidant properties, and may thus contributed to the prevention of infectious, cardiovascular, and cancer diseases ^{13, 23}.

According to Kaliyaperumal A., et al (2013), the plant *Cynodon dactylon* "L" Pers has several medicinal properties such as diuretic, cholagogue, hepatoprotective, anti-inflammatory, antiviral, antibacterial, hypotensive, hypoglycemic, hemostatic, depurative, and astringent ¹⁴.

It can be used in particular ⁸:

- Fight against genito-urinary infections (cystitis, calculations, urolithiasis, urethritis, oliguria, etc.);
- Improve the respiratory system (asthma, bronchitis, etc.)
- Heal superficial wounds
- Or to heal
- Flu in children .
- Conjunctivitis.
- High blood pressure.
- Renal colic .
- Intestinal inflammation.

2.4.1. Antibacterial activity of ingredients :

The compounds with the greatest antibacterial efficacy and the broadest spectrum are phenols (thymol, carvacrol and eugenol). Phenols in particular were useful in bacterial, viral, and parasitic infections whatever their location and caused irreversible lesions on the membranes ⁴⁴.

* Alcohols with 10 carbon atoms (or monoterpenols) come immediately after phenols, in terms of activity, with geraniol, linalool, thujanol, myrcenol, terpineol, and menthol, while piperitol being the best known. Although, broad-spectrum molecules were useful in many bacterial infections ⁴⁴.

*Aldehydes are also somewhat bactericidal. The most commonly used were neral and geranial (from the citrals), citronellal, and cuminal ⁴⁴.

* The most potent antibacterial molecular groups were also effective antifungals, but required longer usage times. Sesquiterpene alcohols and lactones have been found to have antifungal action in basic researches ⁴⁴.

The main objective of the current study was to determine the antibacterial activity of flavonic extracts of plant against six bacterial strains pathogenic for humans.

The disk diffusion method, which involves soaking the disks in 10 µl of each extract, was used to assess the antibacterial activity ³⁸.

Depending on the strains, the following media was used as culture media :

*Mueller-Hinton agar for non-fastidious bacteria.

*Mueller-Hinton agar which contains 5% of horse blood for fastidious bacteria.

The two extracts of acetate and butanol were dissolved in DMSO to obtain decreasing concentrations (C, C/2, C/4, C/8 and C/16) from a stock solution with a concentration equal to 413.5g/l and 1875.975g/l, respectively.

2.4.2. Bacterial strains:

Table 1 : Bacterial strains studied :

Bacterial strains	Characteristics of bacteria	
	The requirement of bacteria	Gram-Type
<i>Escherichia coli</i> : ATCC 25922	Non fastidious bacteria (reference strains)	Gram -
<i>Enterococcus faecalis</i> : ATCC 29212		Gram +
<i>Klebsiella pneumoniae</i> : ATCC 700603		Gram -
<i>Pseudomonas aeruginosa</i> : ATCC 27853		Gram -
<i>Staphylococcus aureus</i> : ATCC 25923		Gram +
<i>Streptococcus pyogenes</i>	Fastidious bacteria (samples (pus) from patients)	Gram +

ATCC : American type culture collection

- **Operating mode :**

The technique of diffusion on solid medium was used as a similar method to that of the antibiogram which involved measuring the sensitivity of bacterial strain to one or more products.

A sterile disc of filter paper (watman n°1) of 6 mm in diameter was impregnated with increasing concentrations of extracts (the different concentrations prepared from two extracts of ethyl acetate and butanol) at a rate of 10µl per disc ²², the latter was then placed on non-inhibitory agar (Mueller Hinton agar and blood agar) poured into standard petri dishes of 4 mm thick and previously inoculated with the selected bacterial strains using a soaked swab in the bacterial inoculum. The concentration of the inoculum was ranged between 10⁶ and 10⁸ C.F.U/ml. The dishes were incubated at a temperature of 37° C. for 18 to 24 hours.

A zone of inhibition or transparent halo formed around the disc if the substance was toxic to the particular species of bacteria. The species was more vulnerable in greater areas.

Table 2: Limit values for the reference strains listed by the National Committee for Clinical Laboratory Standards (NCCLS) and for 10 l of each extract ⁴⁰.

Bacterial strains	Critical values of inhibition diameters (mm)		
	Resistant	Intermediate	Sensitive
<i>Escherichia coli</i> : ATCC 25922	≤12	13 -14	≥15
<i>Enterococcus faecalis</i> : ATCC 29212	≤10	11 -14	≥15
<i>Klebsiella pneumoniae</i> : ATCC 700603	≤12	13 -15	≥16
<i>Pseudomonas aeruginosa</i> : ATCC 27853	≤12	13 -14	≥15
<i>Staphylococcus aureus</i> : ATCC 25923	≤10	11 -15	≥16
<i>Streptococcus pyogènes</i>	≤10	11 -13	≥14

3. Results and Discussion :

3.1. Yields in dry extracts :

The extraction yields were grouped in the following table 1:

Table 3 : Extraction yield

Extract	For 200g of the plant	Yield
Ethyl acetate extract	519,8 mg	0,2599%
Butanol extract	3270,6 mg	1,6353%

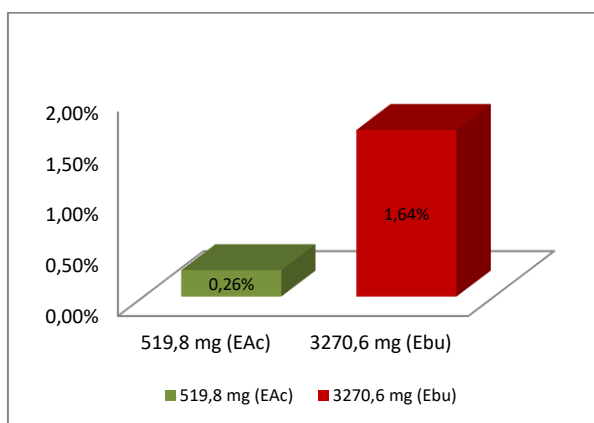


Figure 4 : Yield of extraction

According to the results (Diagram 4), it can be seen that the plant contained 2.559 g/kg of dry extract of ethyl acetate and 16.353 g/kg of dry extract of butanol.

Therefore, the total yield of heterosidic flavonoids in the dry plant was 18.912 g/kg. It can be inferred that it was rich by contrasting it with the *Cynodon dactylon* "L" plant that Daycem Khelifi investigated in 2013 (tanned) ⁵, where he mentioned that the rate of flavonoids was equal to 5.98 g/kg.

Therefore, the total yield of heterosidic flavonoids in the dry plant was 18.912 g/kg. This yield was rich compared to the plant *Cynodon dactylon* "L" studied by Daycem Khelifi in 2013 (tanned) ⁵ where he found that the rate of flavonoids equal to 5.98 g/kg

3.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay:

Absorbance results for different concentrations of both extract and quercetin spread.

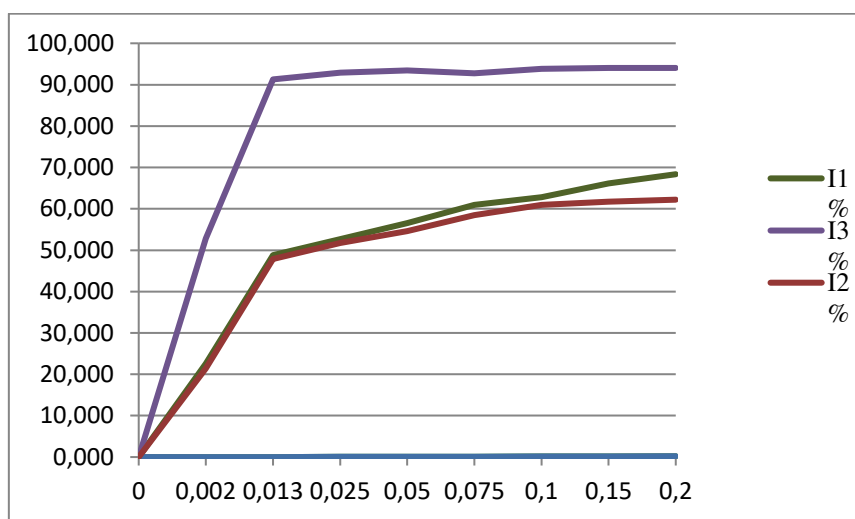


Fig 5 : Evolution of the antiradical activity of plant extracts and Quercetin according to the concentration

I1: for the acetate extract

I2: for the butanol extract

I3: for the quercetin as a reference molecule

FAs shown in figure 5, the concentration of extracts at I50 (IC50), ethyl acetate extract at CI50 $\approx$ 0.014 mg/ml, butanol extract at CI50 $\approx$ 0.019 mg/ml, and reference molecule at CI50 $\approx$ 0 0.0018 mg/ml displayed effective plant activity but not as much as quercetin.

It was found that the activity of the ethyl acetate extract was greater than that of the butanol extract. Nevertheless, it was still lower than that of quercetin.

3.3. Ferric reducing antioxidant power (FRAP) test:

FRAP test assessed the effectiveness of antioxidants in a colored reduction reaction.

The FRAP method of analysis was quick, easy, and reproducible. It was universal can be applied on both plants and Quercetin and organic extracts (Ethyl Acetate and Butanol).

The presence of reducing agents in plant extracts caused the reduction of $\text{Fe}^{3+}/\text{Fe}^{2+}$ ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be evaluated by measuring and monitoring the increase in blue color density in the reaction medium at 700nm.

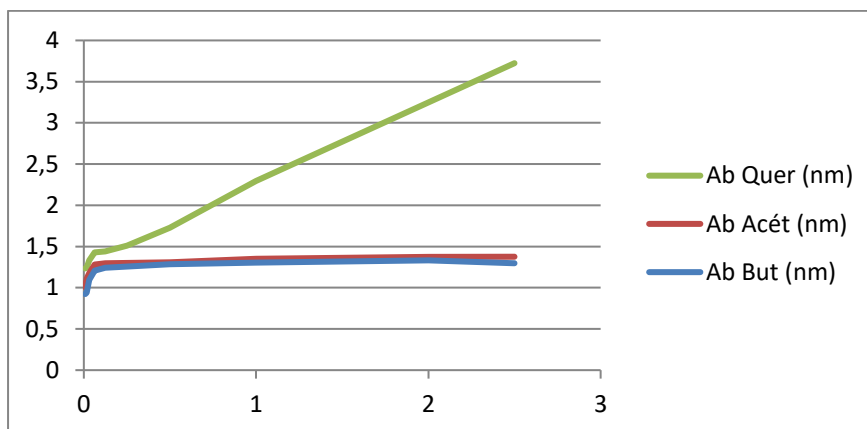


Fig 6 : Reducing power of quercetin, ethyl acetate extract and butanol extract.

According to figure 6, the reducing power at a concentration of 2.5 mg/ml of ethyl acetate extract of *Cynodon Dactylon* was much higher ($Ab=1.376$ nm) compared to the Butanol extract ($Ab= 1.297$ nm) , but significantly lower than that of Quercetin.

The reducing power was due to the presence of hydroxyl groups in the heteroside compounds existing in the extracts that were responsible for reducing of Fe^{+3} ions because they were considered electron donors. This prevented the oxidation reaction of human oxidizing compounds such as oxygen.

3.4. Antibacterial activity :

The diffusion technique on a solid medium involved testing a bacterial strain's reactivity to butanol and ethyl acetate extracts from the *Cynodon dactylon* plant, whose concentrations in DMSO were respectively 413.5 g/l and 1875.975 g/l.

The results of analysis were grouped in the following table 22:

Table 4: Values of the diameters of the zones of inhibition for the reference strains in presence of extracts.

Strains \ Extracts	Ethyl acetate (mm)	Butanol (mm)
Escherichia coli : ATCC 25922	–	10
Enterococcus faecalis : ATCC 29212	13	–
Klebsiella pneumoniae : ATCC 700603	–	08
Pseudomonas aeruginosa : ATCC 27853	–	09
Staphylococcus aureus : ATCC 25923	–	–
Streptococcus pyogènes	14	08

(-) : No zone of inhibition.

This study shown that the bacteria "*Escherichia coli*," "*Klebsiella pneumoniae*," and "*Pseudomonas aeruginosa*" respond well to the butanol extract. The three germs were determined to be resistant strains with regard to the extracts, with the zones of inhibition being, respectively, 10 mm, 08 mm, and 09 mm in comparison to the extract of ethyl acetate which has a negative effect on these germs.

On the other hand, the ethyl acetate extract showed an inhibition zone of 13 mm on the germ of "*Enterococcus faecalis*". As a result, there was a medium inhibition as well as a negative activity for the butanol extract, which implied that the germ was resistant.

Staphylococcus aureus was a germ resistant to the effects of extracts from the *Cynodon dactylon* plant. The *Streptococcus pyogenes* strain exhibited zones of inhibition for both extracts; for ethyl acetate, these zones are 14 mm and for butanol, they are equivalent to 08 mm, indicating that this germ is susceptible to ethyl acetate extract but resistant to butanol extract.

4. Conclusion :

It can be concluded that the glycosides represented 1.8912% of the dry plant while the butanol fraction displayed the highest yield of 1.6353% in relation to the weight of the dry plant.

The ethyl acetate extract has a higher antioxidant activity than that of butanol, according to the results of the two methods used to assess the extracts of the *Cynodon Dactylon* plant (the IC₅₀ of acetate extract was 0.014 mg/ml, while that of butanol was 0.019 mg/ml), which mentioned that aglyconic and monoheterosidic flavonoids have better antioxidant activity than polyheterosidics.

Moreover, research has demonstrated that the functional groups found in phenolic compounds in general were readily capable of donating an electron or a proton to neutralize free radicals ⁴.

Additionally, the presence of hydroxyl groups in phenolic compounds, which can act as an electron donor, was thought to be responsible for the high ethyl acetate extract of *Cynodon dactylon* "L" Pers' species' reducing power ³⁵.

The *Cynodon Dactylon* plant was more active in comparison to earlier research when the IC₅₀ values for the same plant in Tunisia and India were 0.20329 mg/ml and 0.449 mg/ml, respectively ^{5,34}.

The butanol extract displayed a mild antibacterial action against the reference bacterial strains, according to our analysis of the flavonic chemicals found in the two plant extracts. However, only two strains "*Enterococcus faecalis* and *Streptococcus pyogenes*" showed a moderately positive activity to the ethyl acetate extract of flavonoids.

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