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

Given that Cynodon Dactylon "L" Pers is used in traditional medicine for phytotherapy, we are working on evaluating its heteroside extracts in addition to phytochemical screening and antioxidant activity assessment using the "FRAP" method for iron reduction and DPPH radical scavenging. 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 quercitin. The results mentioned that the extract of Ethyl Acetate has a higher activity than that of the Butanol extract "Ethyl Acetate extract IC50 $\simeq$ 0.014 mg/ml, butanol extract IC50 $\simeq$ 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.

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Introduction

Polyphenols are natural compounds widely distributed in the plant kingdom, which have a growing importance, due to their beneficial effects on health [1]. Polyphenols and especially flavonoids are powerful antioxidants capable of inhibiting the formation of free radicals and opposing the oxidation of macromolecules [2]. 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 [3,4]. Flavonoids are powerful inhibitors of oxidation of LDL (Low Density Lipoprotein) [3].

Due to their low redox potential, flavonols (Fl-OH) can thermodynamically reduce free radicals (R), such as superoxyde, peroxyle, alkoxyle, and hydroxyle, by hydrogen transfer; the resulting radical flavonoxy (FL-O·) (shown Figure 1), can then react with another radical to produce a stable quinone structure.

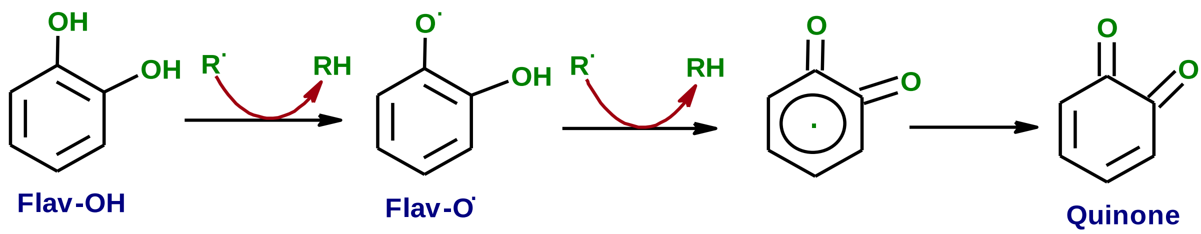

Fig. 1: Trapping of ROS (Reactive oxygen species) (R.) by flavonoids.

Flavonoids are also considered as an effective chelators of metal ions [2,5] such as iron (Fe^{2+}), and copper (Cu^{+}) ions which are essential for certain physiological functions. By reducing hydrogen peroxide, they are also in charge of creating hydroxyl radicals, as demonstrated by the following reaction:

$$HOOH + Fe^{2+}(Cu^{+}) \longrightarrow HO^{\cdot\cdot} + HO^{\cdot} + Fe^{3+}(Cu^{2+})$$

Among the flavonoids that have been studied, quercetin is the most active. Figure 2 provides a summary of the key sites for metal ion chelation. The B ring has a catechol ring, the C ring has 3-hydroxyl and 4-oxo groups, and the region between rings A and C has 5-hydroxyl and 4-oxo groups [2].

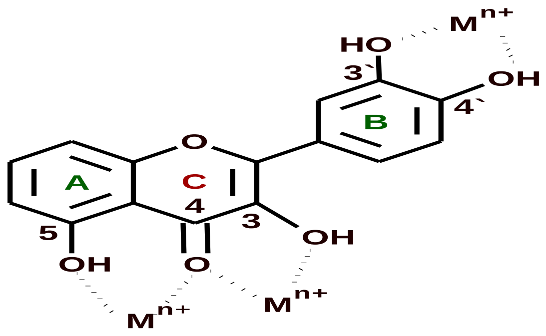

Fig. 2: Flavonoids and their sites proposed for chelation metal ions (Mn+)

(From Van Acker S., and al 1995) [2]

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 [6].

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 transcriptas [7,8,9]. Moreover, various studies have provided evidence of the bactericidal effect of different flavanones on the DNA gyrase from a *Staphylococcus aureus bacterium* [10].

Naturally, flavonoids are very often found in the form of glycosides, where there are two types of heteroside flavonoids [11].

• O-heteroside

• C-heteroside

In general, the hydroxyls in position "7" of flavones and position "3" of flavanols—which are engaged in the case of O-glycosil—can perform the bond between genin and ose.

More than 300 C-heterosides have been identified; they form a bond between the anomeric carbon of sugar and the carbon at position 6 or 8 of the genin. [12]

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 wild 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 on 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 [13].

Despite its bad image, Greek doctors have long used quack grass as a diuretic and kidney stone remover due to the potassium salts contained in its stem and rhizome. It is also used for its febrifuge properties. There have been reports of quack grass acting as an emollient and preventing urinary issues like cystitis and urethritis. It safeguards the urinary system.

In decoction, it relieves inflammation of the prostate. Further,ore, Quack grass prevents the recurrence of renal colic served as an infusion, it fights against cellulite [13].

Also, C.dactylon leaf extract has been reported to have antidiabetic, [14,15], antioxidant, hypolipemia, [5,6,16] healing of minor injuries [17], and hepatic antioxidant properties [18].

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 m^2, 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 [19].

The inflorescence is formed up of two to six straight, purple or green digitate ears with a villous rachis at the base. The ears are arranged in two close rows and appear toothed, with a maximum of 50 mm in length. The flowering season varies from February to November [19,20].

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.

Materials and Methods

1. Extraction and determination of flavonic glycosides

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

By macerating and stirring plant material for 24 hours in an ethanol/water mixture (80/20: V/V), the ethanoic extracts were produced. The solvent/plant material ratio was 10/1 (ml/g) [21].

Rota-steam was used to concentrate the extract at 40°C and 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 (3x200 ml), the butanol phase was evaporated to dryness at 40°, and the residue was recovered with 10 ml of methanol.

The butanol extract contains poly heterosides and C-glycosides, whereas the ethyl acetate extract contains mono heterosides, aglycones, and occasionally diheterosides [5].

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) [22].

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

$\mathbf{Y_t}$: Total yield.

$\mathbf{W_1}$: Flask weight after evaporation. .

$\mathbf{W_2}$: Weight of empty flask.

$\mathbf{W_3}$: Weight of the starting plant material.

3. Activité anti oxydante of flavonoid heterosides

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 [23,24]. It has an unpaired electron on an atom of the nitrogen bridge (shown Figure3). DPPH is a purple free radical that turns yellow when reduced by an H atom donor.

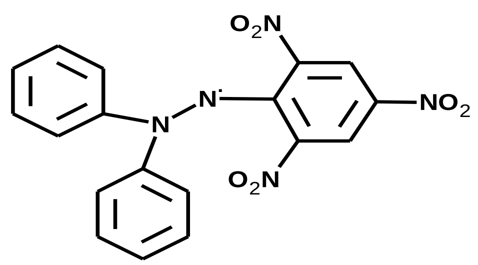

Fig. 3: The free radical DPPH• (2,2 DiPhenyl-1-Picryl-Hydrazyl)

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) [25, 26].

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 [3,5].

$$\mathbf{DPPH^{\cdot} + PhOH \longrightarrow DPPHH + PhO^{\cdot}}$$

- *Operating mode*

A range of concentrations (0-200 μg/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:

$$I\% = (1 - \left(\frac{\mathrm{DO_{essai}}}{\mathrm{DO_{b}}_{lanc}}\right)) \times 100$$

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.

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 $K_3Fe(CN)_6$. 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 [28].

- *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 $K_3Fe(CN)_6$. 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 [29].

4. Antibacterial activity of flavonoid heterosides

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 [30,31].

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 [13]. It can be used in particular [32]:

- 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.

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 [33].

* 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 [33].

*Aldehydes are also somewhat bactericidal. The most commonly used were neral and geranial (from the citrals), citronellal, and cuminal [33].

* 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 [33].

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 (shown Table 1).

The disk diffusion method, which involves soaking the disks in 10 µl of each extract, was used to assess the antibacterial activity [34].

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.

4.2. Bacterial strains

Table 1 present the bacterial strains and the caracteristics of the bacteria.

Table 1 : Bacterial strains studied

Bacterial strains	**Characteristics of bacteria**	
	The requirement of bacteria	**Gram-Type**
Escherichia coli : ATCC 25922	Non fastidious bacteria (reference strains)	Gram -
Enterococcus faecalis : ATCC 29212		Gram +
Klebsiella pneumoniae : ATCC 700603		Gram -
Pseudomonas aeruginosa : ATCC 27853		Gram -
Staphylococcus aureus : ATCC 25923		Gram +
Streptococcus pyogenes	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 [35], 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 between106 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. We compare with the table 2 data which contains the Limit values for the reference strains listed by the National Committee for Clinical Laboratory Standards (NCCLS) (national Committee for clinical laboratory standards), for 10 µl of each extract [36].

Table 2: Limit values for the reference strains listed by the National Committee for Clinical Laboratory Standards (NCCLS) for 10 µl of each extract [36].

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

Results and Discussion

1. *Yields in dry extracts*

The extraction yields were grouped in the following table 3 and Figure 4:

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%

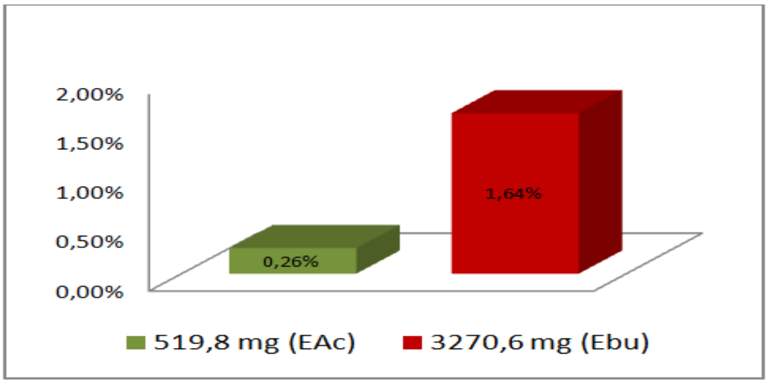

Fig. 4: Yield of extraction

According to the results shown in the table 3 and Figure 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 Khlifi investigated in 2013 (tanned) [37], 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 Khlifi in 2013 (tanned) [37], where he found that the rate of flavonoids equal to 5.98 g/kg.

2. Activité anti oxydante of flavonoid heterosides

2.1. *2,2-diphenyl-1-picrylhydrazyl (DPPH) assay*

Absorbance results for different concentrations of both extract and quercetin spread (shown in Figure 5).

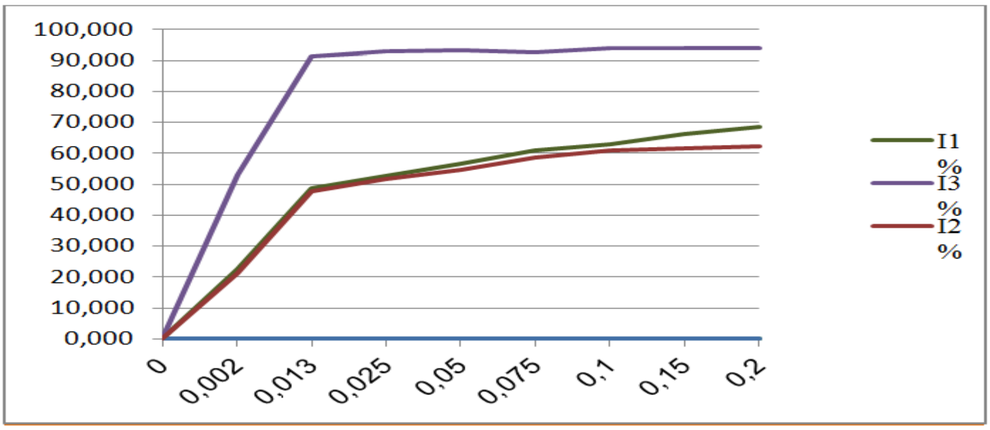

Fig.5 : Evolution of the antiradical activity of plant extracts and Quercetin according to the concentration

I1: for the acetate extract

I2: for the butanol extract

I3: for the quercitin as a reference molecule

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

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.

2.2. *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 Fe^{3+}/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.

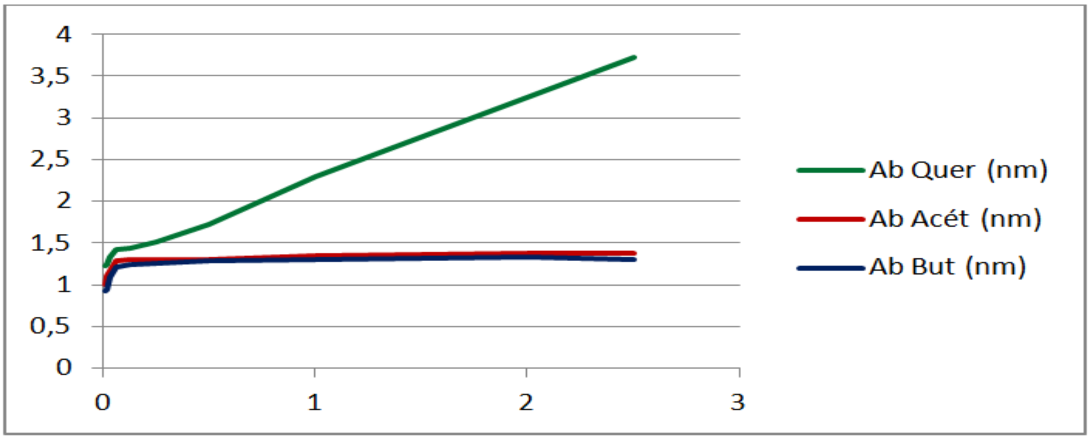

Fig. 6: Reducing power of quercetin, ethyl acetate extract and butanol extract.

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. Antibacterial activity of flavonoid heterosides

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 4:

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

Extracts Strains	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.

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 [38].

Additionally, the presence of hydoxyl groups in phenolic compounds, which can act as an electron donor, was thought to be responsible for the high ethyl acetate extract of Cynodon dacylon "L" Pers' species' reducing power [39].

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 [6,37].

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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