Phytochemical content and antioxidant activity of *Calycotome Spinosa's* aqueous and hydro-ethanolic extracts using conventional and unconventional extraction methods

ABSTRACT

This study was carried out to assess the main secondary metabolites contents and antioxidant activity of aqueous and hydro-ethanolic extract of *Calycotome Spinosa* plant using either conventional (maceration, reflux, and Soxhlet) or unconventional (Microwave-Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE)) methods. The highest extract yields were recorded for MAE methods in both water (18.15%) and ethanol extraction (21.37%) respectively. MAE method showed the highest rate of total phenols (168.24±0.79 and 182.60±1.29 mg GAE/g DR) and total flavonoids (16.38±1.17 and 28.94 ±0.67 mg GAE/g DR) contents in both water and hydro-ethanol respectively. While, the highest tannin content was recorded in maceration and MAE methods (18.90±2.82 and 23.01±2.20 mg CE/g DR) in aqueous and hydro-ethanolic extracts respectively. MAE method exhibited a significant ability to scavenge DPPH radical (IC50= 0.51 ± 0.39, and 0.34 ±0.48 mg/ml) in both water and hydro-ethanol respectively. We conclude that MAE was more effective as an extraction method for *C. Spinosa* plant which allows a good extraction yield with a high rate of secondary metabolite and a high antioxidant activity.

Keywords: *Calycotome Spinosa*, secondary metabolites, conventional methods, unconventional methods, antioxidant activity.

1. Introduction

Reactive oxygen species (ROS) are formed by living organisms as a result of natural cellular metabolism and environmental causes, such as air pollution or tobacco smoke. ROS are highly reactive molecules that can destroy cell structural molecules such as sugars, nucleic acids, lipids, and proteins and alter their functions (Birben, Sahiner et al. 2012). The development of ROS is related to antioxidant protective mechanisms in stable aerobic organisms (Halliwell 2007). The change in the balance in favor of oxidants between oxidants and antioxidants is called "oxidative stress" (Birben, Sahiner et al. 2012). The development of many neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Aamyotrophic Lateral Sclerosis (ALS), oxidative stress is highly involved (Barnham, Masters et al. 2004). Growing research suggests that in the development of metabolic disorders such

as diabetes and cardiovascular diseases, chronic and acute overproduction of ROS under pathophysiological conditions is important (Madamanchi, Vendrov et al. 2005). It is also recognized that ROS can cause cell membrane instability (Mora, Paya et al. 1990), DNA structure destruction ,and mutations induction (Sastre, Pallardó et al. 2000; Sato, Kato et al. 2001), Infertility and carcinogenic effects have also been observed (Kawanishi, Hiraku et al. 2001; Sheweita, Tilmisany et al. 2005). However, several synthetic antioxidants have been commonly used in various food products, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate. However, their use as food additives is tightly limited in many countries because of possible health hazards (Wang, Shao et al. 2009). Many experiments are currently focused on discovering and using natural antioxidants to reduce harmful free radicals in the human body, thus avoiding or curing diseases (Li, Li et al. 2014). Local flora is full of many varieties of plants that have actual pharmacological properties, which consist on a natural reservoir of bioactive molecules still poorly studied (Bentabet, Boucherit-Otmani et al. 2014; El Guiche, Tahrouch et al. 2015). Complete and perfect monitoring of the numerous properties of these plants, including the recognition of all physicochemical classes capable of inducing one or more pharmacological effects, is now a goal of first order. *Calycotome spinosa* or "Guendoul" as a local vernacular name belongs to the family of Papillionaceae (Fabaceae); during the spring season it is a spiny arbuste, trifoliate with yellow flowers, widespread in the Mediterranean undergrowth forest and prefers well-watered silica soils (Cherfia, Ali et al. 2017). While this plant is abundant in bioactive metabolites, negligible metabolites remain. In addition, in Sicilian folk medicine, the aerial component of this genus, Calycotome, is commonly used as an antitumor agent as well as for the treatment of furuncles, cutaneous abscess, and chilblain (Cherfia, Zaiter et al. 2020). The infusion of this plant with flowers is also used by the Palestinian people to treat cardiovascular and nervous system disorders (Alzweiri, Al Sarhan et al. 2011). The foliage of this plant is also very rich in crude protein (33.7 %), which makes it an excellent protein substitute for fibrous products of poor quality forage and undergrowth. Fortunately, phenols and tannins are disproportionately rich (Spínola, Pinto et al. 2015). In addition, several compounds were obtained and classified from Calycotome genus extracts, such as phenolics, flavonoids, alkaloids, and anthraquinones (Loy, Cottiglia et al. 2001). To our knowledge, this is the first time that a comparison between conventional and unconventional extraction methods has been made for stems extracts of *Calycotome spinosa*.

1. Materials and methods

1.1.Plant sampling

Phytochemical study and evaluation of antioxidant activity required plant material represented *Calycotome spinosa* or known by "Guendoul" as a local vernacular name, harvested in "El Kheiter" in the region of El Bayadh in Algeria during the month of February 2019 (Latitude: 34.1434, Longitude: 0.0732471 34 ° 8 ′ 36 ″ North, 0 ° 4 ′ 24 ″ East). The plant used was identified by Dr. Eddoud Amar from the Department of Biological Sciences of Kasdi Merbah at Ouargla University. The dry stems of plant was ground and used for the preparation of the various extracts **(Figure 1).**

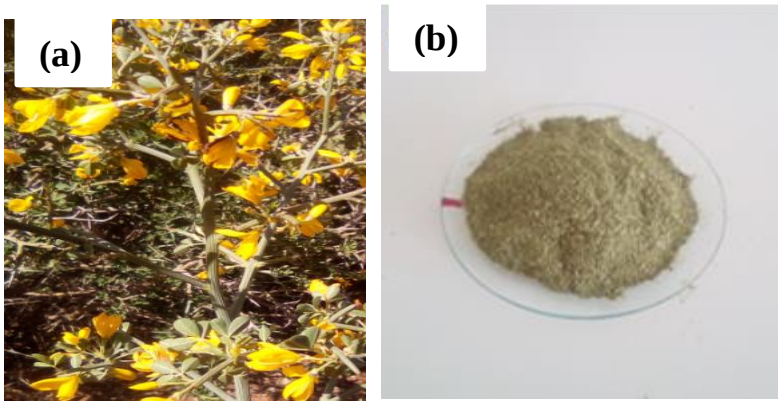


Fig. 1. The appearance of the aerial part (a),
and dry grounded steams of *Calycotome spinosa* (b).

1.2.Plant extracts preparations

Extracts were obtained both with conventional methods, as maceration, reflux and soxhlet, and using the unconventional ones as Microwave-Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE). 10 g of powder was weighed for each extraction and two solvents (water and hydro-ethanol 70%) were measured into a 250 mL conical flask depending on the feed-to-solvent ratio (1:10 g/mL). Time extraction processes was about 48h for maceration, 4h for reflux and soxhlet, 10 min for MAE (2450kHz) and 30 min for UAE (35kHz). The extract solutions were filtered through a cone of filter paper (Whatman no 1), concentrated to dryness using a rotary evaporator, and stored at 4°C until use. The extraction yield was calculated using the following formula:

$$\text{Yield of extract (\%)} = \left(\frac{\text{Weight of extracts from plant sample}}{\text{Weight of dried plant sample}}\right) \times 100\%$$

1.3.Secondary metabolites contents and antioxidant activities

Total Phenolic Content (TPC) estimation was carried out by means of Folin-Ciocalteu's reagent, following the (Serairi-Beji, Aidi Wannes et al. 2018) method. The reference standard was gallic acid (GA). Results are expressed as mg GA equivalents (GAE)/g dry residue. The total flavonoid content (TFC) of the extracts has been determined using the colorimetric method as described by (Kim, Kang et al. 2013). TFC was expressed as mg catechin (C) equivalent per gram dry weight (mg CE/g DR). Total condensed tannin (TCT) was measured according to the method described by (Serairi-Beji, Aidi Wannes et al. 2018). TCT was expressed as milligrams catechin equivalent per gram dry weight (mg CE/g DR). The antioxidant activity was investigated employing the $DPPH^{\cdot}$ assay, following the method proposed by (Sánchez-Moreno, Larrauri et al. 1998) ,and ferric reducing antioxidant power (FRAP) assay, assessed by means of potassium ferricyanide-ferric chloride method described by (Mahmoudi, Khali et al. 2013).

1.4.Statistical analysis

All assays were carried out in triplicate (n=3) and their results were expressed as mean ± standard error of the mean and analyzed by SigmaPlot for Windows version 11.0. A comparison between groups was made using the Tukey-test. Columns not sharing a common letter (a–c) differ significantly at $p < 0.05$ (Tukey-test).

2. RESULTS

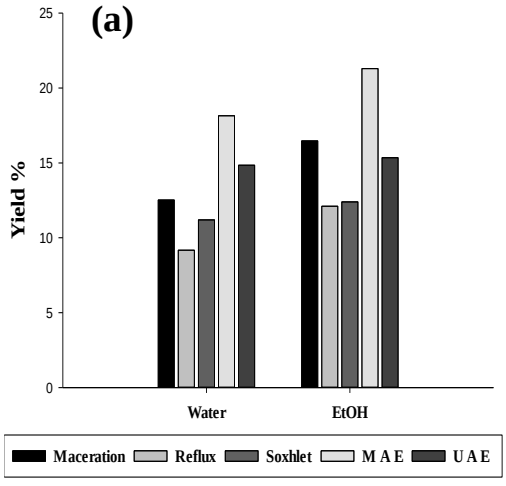
2.1. Extraction yield

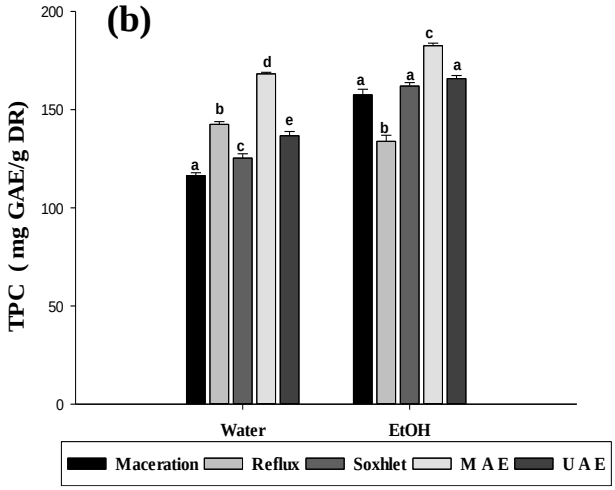
The extraction of the stems of *Calycotome spinosa* by means of five extractions methods using two solvents allows us to calculate the extraction yields expressed in percentages relative to the initial dry weight. As shown in **figure 2-a**, the highest yields in water extraction were obtained in MAE method (18.15%), followed by UAE method (14.85%), then maceration (12.53%), Soxhlet (11.2%), and reflux (9.17%). While, the highest yields in hydro-ethanol extraction were obtained in MAE method (21.37%), followed by maceration (16.47%), then UAE method (15.34%), Soxhlet (11.40%), and reflux (12.11%).

2.2.Total phenol, flavonoid, and condensed tannin contents

The highest total phenol rate in water extraction were observed in MAE methods (168.24±0.79 mg GAE/g DR) followed by reflux, UAE method, Soxhlet, and maceration (142.49 ±1.41, 136.74 ±2.18, 125.36 ±2.2, and 116.40 ±1.49 mg GAE/g DR respectively).

While, the highest TPC in hydro-ethanol extraction were observed in MAE methods (182.60±1.29 mg GAE/g DR) followed by MAE method, Soxhlet, maceration and reflux (165.82 ±1.59, 162.03 ±1.76, 157.55 ±2.87, and 133.87 ±3.12 mg GAE/g DR respectively) (**figure 2-b)**. However, significant high level of flavonoids content in water extraction were observed in MAE methods (16.38±1.17 mg CE/g DR), followed by UAE method, Soxhlet, maceration and then reflux (14.84±1.93, 12.53±2.03, 12.02±1.11, and 10.74±1.55 mg CE/g DR respectively). While, the highest TFC in hydro-ethanol extraction were observed in MAE methods (28.94 ±0.67 mg CE/g DR) followed by UAE method, maceration, reflux, Soxhlet, and then reflux (25.61 ±1.93, 21.51 ±0.92, 19.71 ±1.84, and 17.41 ±1.35 mg CE/g DR respectively) (**figure 2-c)**. In addition, the highest levels of condensed tannins were recorded in water extraction for maceration (18.90±2.82 mg CE/g DR) followed by MAE and UAE methods, Soxhlet, and then reflux (15.76±2.37, 13.6±3.56, 10.27±2.07 and, 8.9±2.04 mg CE/g DR respectively). While, the highest TTC in hydro-ethanol extraction were observed in MAE (23.01±2.2mg CE/g DR) followed by maceration, reflux, UAE method, and soxhlet (20.07±1.19, 17.33±3.4, 17.33±3.4, and 14.39±3.74 mg CE/g DR respectively) (**figure 2-d)**.





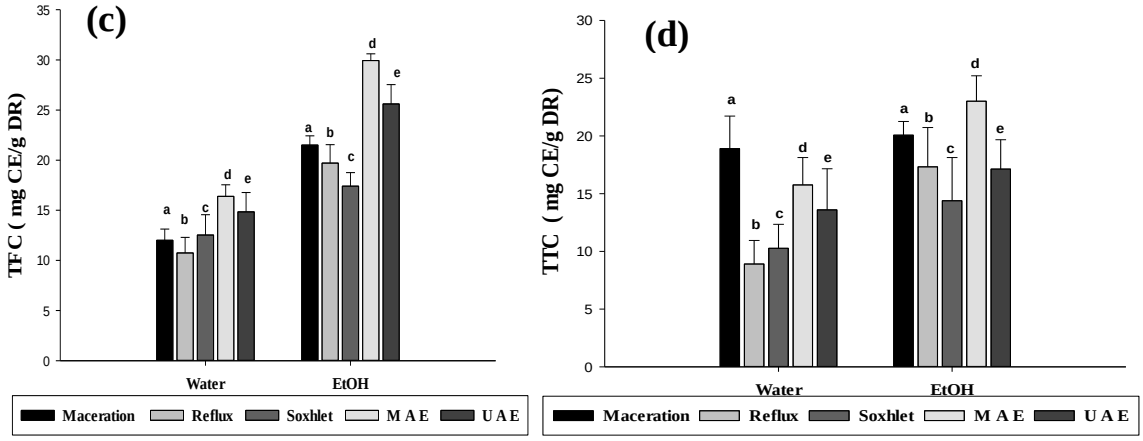


Fig. 2. (a) Extraction yield, (b) Total phenolic content (TPC), (c) Total flavonoid content (TFC) , and (d) Total tannin content (TTC) of the different extracts from *Calycotome Spinosa*. Data are expressed as means±SEM (n=3). Comparison between groups was made using the Tukey-test. Columns not sharing a common letter (a–e) differ significantly at $p < 0.05$ (Tukey test).

2.3.Antioxidant activities

Two complimentary tests were used in this study to assess the antioxidant activity of the different extracts from *Calycotome Spinosa*: DPPH free radical-scavenging activity and reducing power (FRAP) assays. Results expressed in figure 3 and table 1 show that MAE exhibited a high significant ability to scavenge DPPH radical (IC50= 0.51 ± 0.39 mg/ml) in water extraction followed by Soxhlet, reflux, UAE and maceration (0, 51 ± 0.39, 0.69 ± 0.47, 0.75 ± 0.72 and 0.83 ± 0.62 mg/ml respectively). While, in hydro-ethanol extraction MEA method exhibited a high significant ability to scavenge this radical (0.34 ±0.48 mg/ml) followed by maceration, UEA, Soxhlet, and reflux (0.48 ±0.33, 0.67 ±0.41, 0.69 ±0.48, and 0.71 ±0.56 mg/ml respectively). In the other hand, maceration in water extraction shows a significant iron reducing power (EC50= 1.225±0.001 mg/ml) followed by Soxhlet, UEA, reflux, and MEA (1.086±0.002, 0.962±0,001, 0.952±0.002, and 0.634±0.002 mg/ml respectively). While, reflux extraction in hydro-ethanol solvent exhibited a significant iron reducing power (EC50= 1,144±0,001 mg/ml) followed by maceration, UEA, Soxhlet, and MEA (0,897±0,088, 0,837±0,002, 0,836±0,003, and 0,459±0,001 mg/ml respectively).

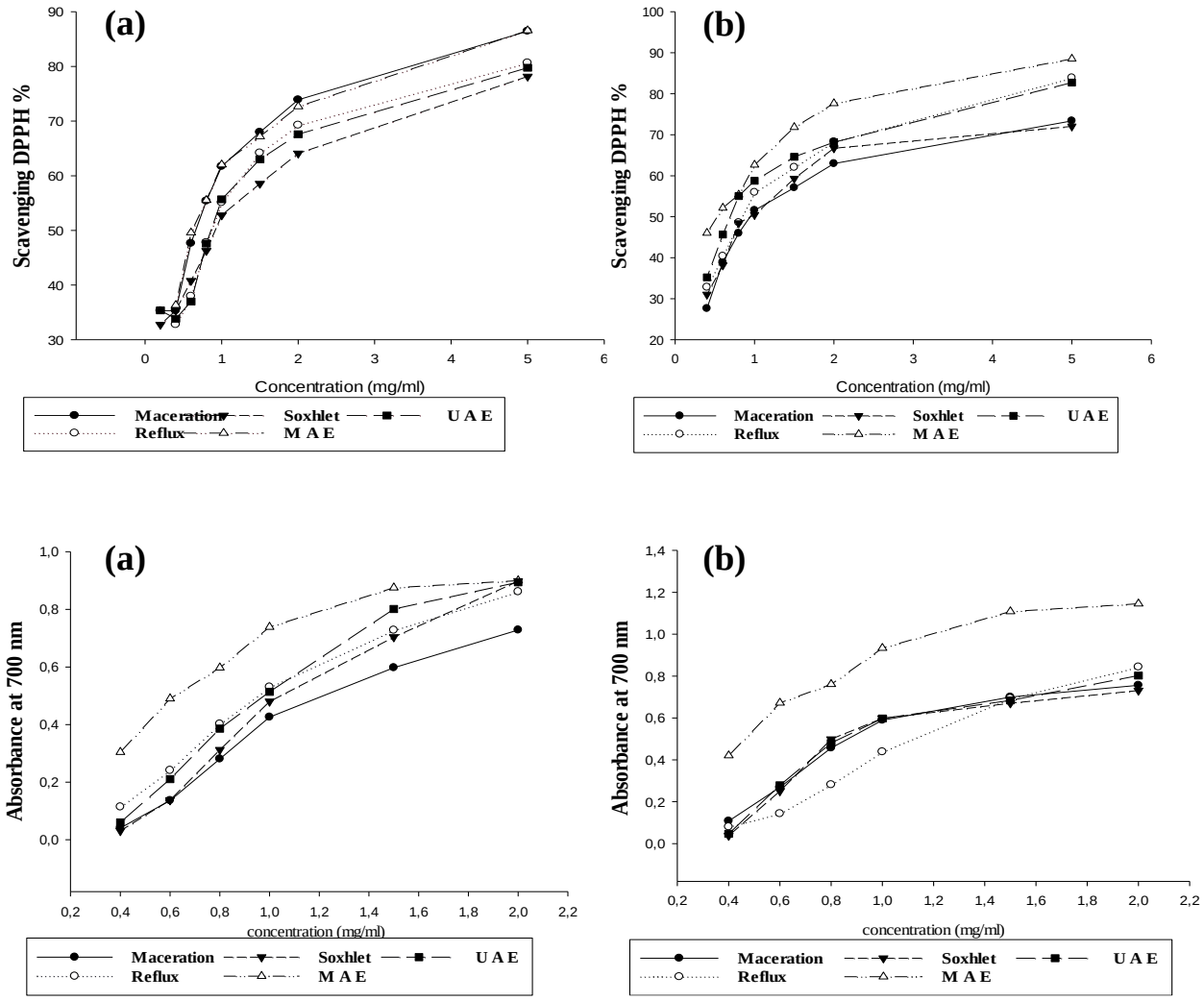


Fig. 3. (a-b) DPPH radical-scavenging activity for water and ethanol extracts; and (c-d) Ferric reducing antioxidant power assay (FRAP) of water and ethanol extracts. Data are expressed as means±SEM (n=3).

Table 1. DPPH radical-scavenging activity and Ferric reducing antioxidant power assay of the different extracts from *Calycotome Spinosa*. Data are expressed as means±SEM (n=3). Comparison between groups was made using the Tukey-test. Column not sharing a common letter (a–c) differ significantly at $p < 0.05$(Tukey-test).

	DPPH Means IC_{50} (mg/ml)±SEM		FRAP Means EC_{50} (mg/ml)±SEM	
	H_2O	EtOH	H_2O	EtOH
macération	0, 83 ± 0,62 [a]	0, 48 ±0,33 [a]	1,225±0,001 [a]	0,897±0,088 [a]
reflux	0, 69 ± 0,47 [b]	0, 71 ±0,56 [b]	0,952±0,002 [b]	1,144±0,001 [b]
Soxhlet	0, 51 ± 0,39 [c]	0, 69 ±0,48 [b]	1,086±0,002 [a]	0,836±0,003 [c]
M E A	0, 47 ± 0,76 [c]	0, 34 ±0,48 [c]	0,634±0,002 [c]	0,459±0,001 [d]
U E A	0, 75 ± 0,72 [b]	0, 67 ±0,41 [b]	0,962±0,001 [b]	0,837±0,002 [c]

3. DISCUSSION

Nowadays, because of their antioxidant activities, there is growing exposure to the health benefits of plant phenolic compounds (Serairi-Beji, Aidi Wannes et al. 2018). This study was conducted to characterize the phenolic profile and antioxidant potential of the extracts obtained from *Calycotome Spinosa* using two solvents and five methods belonging to conventional and unconventional extraction methods namely: maceration, reflux, Soxhlet, Microwave-Assisted Extraction (MAE), and Ultrasound-Assisted Extraction (UAE). To our knowledge, no research has yet been done on the unconventional extraction of *C. spinosa* stems. Our results show that the highest extract yields were recorded for MAE methods in both water (18.15%) and ethanol extraction (21.37%) respectively followed in descending order by UAE method, maceration, Soxhlet and reflux. Our finding agree with those of (Aspé and Fernández 2011) who estimated the performance of four techniques, conventional maceration, Soxhlet extraction, microwave assisted extraction (AEM) and ultrasound assisted extraction (WATER), for bark extraction of *Pinus radiata,* and found that the extraction mass increased in the following order: Soxhlet, MAE, UAE and maceration. In addition, the study of (Karami, Emam-Djomeh et al. 2015) conducted in order to optimize the extraction condition of secondary metabolites by microwave application, found that the MAE was more efficient extracting method than Soxhlet. Furthermore, MAE method showed the highest rate of total phenols (168.24±0.79 and 182.60±1.29 mg GAE/g DR) and total flavonoids (16.38±1.17 and 28.94 ±0.67 mg GAE/g DR) contents in both water and ethanol respectively. While, the highest tannin content was recorded in maceration and MAE methods (18.90±2.82 and 23.01±2.20 mg CE/g DR) in aqueous and hydro-ethanolic extracts respectively, followed in descending order by UAE method, Soxhlet, maceration, and reflux. Nevertheless, our results remain higher than the results found by (Cherfia, Ali et al. 2017) who found in *Calycotome Spinosa*, after using conventional extraction method, a polyphenol contents of 107.75±0.41 and 64.24±1.81 mg gallic acid equivalents/g extract for leaves ethyl acetate and n-butanol respectively and 81.45±0.6 and 96.06±2.72 mg gallic acid equivalents/g extract for flowers ethyl acetate and n-butanol successively. The total phenolic and flavonoid contents vary according to the plant organ used, the species analyzed, and the choice of solvent and extraction methods (XU and CHANG 2007).
An imbalance in the oxidant/antioxidant status is often followed by tissue injury and human d sease, causing oxidative stress, which must be amenable to therapeutic intervention with suffi

cient antioxidants, provided that they can enter the site of damage and are successful in decre sing oxidative damage levels (Halliwell 2001). Therefore, because of the possible health risks of many synthetic antioxidants commonly used in various food products that include toxic side effects, their usage in many countries is strictly controlled (WANG *et al.* 2009). That is why there is a rising interest in substituting synthetic antioxidants by natural ones for food preservation (Serairi-Beji, Aidi Wannes et al. 2018). In reality, polyphenols are natural compounds that are widely distributed in the plant kingdom and are increasingly essential, particularly because of their beneficial health effects (Koechlin-Ramonatxo 2006). The antioxidant activity of polyphenols is largely attributable to their redox properties, which make them act as reducing agents, hydrogen donors, singlet oxygen quenchers, and even potential metal chelators (Adebiyi, Olayemi et al. 2017). In this study, two complementary tests were used to assess the antioxidant activity of *Anvillea radiata* (DPPH free radical-scavenging activity, and ferric reducing antioxidant power assay). The results showed that MAE method exhibited a significant ability to scavenge DPPH radical (IC50= 0.51 ± 0.39, and 0.34 ±0.48 mg/ml) in both water and ethanol respectively, and a significant iron reducing power (EC50= 1.2254±0.0017 and 1.1441±0.0018 mg/ml) were recorded for maceration and reflux methods in aqueous and ethanol extracts respectively. We found that MAE was more effective as an extraction method for *C. Spinosa* plant which allows high antioxidant activities. (Cherfia, Ali et al. 2017) in order to assess the antioxidant activity of the ethanol extract of the aerial parts of *Calicotome villosa subsp*, and shows that the ethanol extract reduced the DPPH radical formation IC50 value of 68 µg/mL. Moreover, Similar results were found in a study carried out on another species of the genus Calycotome and which shows a significant ability to scavenge DPPH radical (IC50 = 0.20mg / ml) in methanolic extract of *Calycotome villosa subsp. Intermedia* (Elkhamlichi, El Hajaji et al. 2017).Our results do not deviate from the study carried out by (Nayak, Dahmoune et al. 2015) during the comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from *Citrus sinensis* peels, and they found that the total phenolic content (TPC), total antioxidant activity (TAA) (using DPPH and ORAC-values) and individual phenolic acids (IPA) were higher than the other three extracts in MAE extracts. Another study carried out to compare between two different extractive techniques in order to get qualitative and quantitative data regarding bioactive compounds of four different spices, concluded that the efficiency of extraction of bioactive compounds obtained with the microwave extraction process was in general about four times higher than that resulting from sonication extraction (Gallo, Ferracane et al. 2010). (Aspé and Fernández 2011) state that MAE was a simple and

rapid method that was useful for extraction of *P. radiata* bark and and declare that MAE extracts presented a higher anti-radical capacity than does Soxhlet. In the same context (Hayat, Hussain et al. 2009) declare that MAE could be a fast and reliable method for quantitative analysis of phenolic compounds in their study carried out to optimized microwave-assisted extraction of phenolic acids from citrus mandarin peels and evaluation of their antioxidant activity in vitro.

4. Conclusion

The results of the present investigation indicate that the highest extract yields were recorded for MAE. This same method showed the highest rate of total phenols, total flavonoids and the highest tannin content. MAE exhibited also a high ability to scavenge DPPH radical. From these data we conclude that MAE is more effective as an alternative method to conventional ones for the extraction and exploitation of secondary metabolites of *Calycotome Spinosa*.

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