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EVALUATION OF ECO-EXTRACTION METHODS OF ANTIOXIDANTS AND THEIR ACTIVITIES FROM *RETAMA RAETAM* TWIGS

***Oussama Zaoui*[1], *Karima Oughlissi-Dehak*[2], *Mebarka Bouziane*[3], *Fatiha Zaoui*[4], *Farouk Boudou*[5], *Amina Benras*[6], *Abdelghani Sehmi*[7], *Mahfoud Hadj-Mahammed*[8]**

[1] ORCID: 0000-0002-5975-2691
[2] ORCID: 0009-0003-4128-6316
[3] ORCID: 0000-0002-7359-8555
[4] ORCID: 0000-0002-1651-5194
[5] ORCID: 0000-0002-6008-3325
[6] ORCID: 0000-0003-1231-7905
[7] ORCID: 0000-0002-3108-8362
[8] ORCID: 0000-0003-0345-0798
[1–3,6,8] Biogeochemistry of Desert Environments Laboratory,
Mathematics and Matter Sciences Faculty
University Kasdi Merbah-Ouargla, Algeria
[4] Laboratory of Separation and Purification Technologies, Chemistry Department,
Faculty of Sciences
Tlemcen University, Tlemcen, Algeria
[5] Research Laboratory of Environment and Health (RLEH), Faculty of Medicine,
Sidi-Bel-Abbes, Algeria
[7] Department of Chemistry, Faculty of Sciences
University of Saïda, Saïda, Algeria

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Abstract

The purpose of this study is to compare the effectiveness of different conventional and non-conventional methods for the extraction of secondary metabolites and antioxidant activity of aqueous and hydro-ethanolic extract of *Retama raetam* twigs including maceration, reflux, Soxhlet, microwave assisted extraction (MAE) and ultrasonic assisted extraction. The aqueous and hydro-ethanolic extracts obtained by MAE showed the highest contents of total phenolics (160.43 ±1.42 and 175.71 ±2.09 mg EAG/g DR, respectively) and flavonoids of 12.28 ±0.92 and 39.97 ±1.11 mg EC/g DR, respectively. It also exhibited significant DPPH˙ scavenging capacity with IC_{50} values of 0.45 ±0.075 and 0.34 ±0.039 mg/mL and significant iron reducing capacity with EC_{50} of 0.358 ±0.02 and 0.28 ±0.01 mg/mL for the aqueous and hydro-ethanolic extracts, respectively. The MAE proved to be the most efficient extraction technique for the extraction of antioxidants from *R. raetam* twigs.

Address: Oussama Zaoui, University Kasdi Merbah-Ouargla, Ouargla 30000, Algeria, phone: +213773348978, e-mail: zaouioussama@outlook.fr

Introduction

Secondary metabolites, such as phenolic components, are commonly found in plants and have a wide range of structures. Furthermore, these compounds are not uniformly distributed in plants and have varying degrees of stability. This has resulted in challenging extraction processes, meaning that the use of a single step or an ineffective extraction approach can affect the recovery of phenolic compounds from plant samples. To recover the desired phenolic components, it is critical to select a suitable extraction procedure. These approaches encompass both conventional and non-conventional ways of extraction (ALARA and ABDURAHMAN et al. 2021). However, microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) are among the non-conventional green chemistry (GC) extraction methods (ZIN and ANUCHA et al. 2020). GC is the need of today and the light of the future which gives a precious idea for scientifically based environmental protection (ASIF and IMRAN 2021). It is a fascinating research area due to its respect for the environment (MENGES 2021) GC involves a reduction or elimination of the use of hazardous substances in a chemical process or the generation of hazardous or toxic intermediates or products. This includes feedstock, reagents, solvents, products, and byproducts. It also includes the use of sustainable raw materials and energy sources for this manufacturing process (DOBLE and KRUTHIVENTI 2007). MAE is considered a new method of extracting fluid soluble products from many materials using microwave energy (REHMAN and KHAN et al. 2020). it is an automated green extraction technique that offers many advantages such as faster heating to extract bioactive materials from matrices, smaller equipment size, reduced thermal gradients and the possibility to extract several samples simultaneously, thus considerably improving the sample throughput and increasing extract yield (LLOMPART and CELEIRO et al. 2019). The extraction time of bioactive compounds in the case of MAE is lower than conventional extraction methods. In addition, it is a selective process for the extraction of organic and organometallic substances (REHMAN and KHAN et al. 2020). While, ultrasound-assisted extraction is another effective technique that has become more popular since 2007 (REDDY and MONIRUZZAMAN et al. 2020). UAE has been considered a promising and innovative technique with many applications in the chemistry, pharmaceutical, cosmetic, and alimentary fields of the 21st century (CHAHARDOLI and JALILIAN et al. 2020). It is also used in the search for bioactive compounds as it is based on the effects of acoustic cavitation. The propagation of ultrasonic waves allows greater penetration of the solvent into the sample matrix, which increases the contact between the sample

and the solvent and improves mass transfer rates (DUARTE and JUSTINO et al. 2014). To the best of our knowledge, no study has been conducted on the extraction of secondary metabolites from the *R. raetam* plant using GC techniques. Therefore, the present study was conducted to investigate the efficiencies of MAE and UAE methods as innovative and eco-friendly technology using two green solvents compared to conventional extraction techniques such as maceration, reflux and Soxhlet for the extraction of major secondary metabolites and evaluation of their antioxidant activities.

Materials and Methods

Chemicals and reagents

Analytical grade ethanol, 2-(3,4 Dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (Catechin; C), 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 3,4,5-trihydroxybenzoic acid (Gallic acid; GA), 3-methoxy-4-hydroxybenzaldehyde (Vanillin), aluminum chloride ($AlCl_3$), ammonium molybdate ($(NH_4)_6Mo_7O^2_4$), Folin-Ciocalteu phenol reagent, hydrochloric acid (HCl), iron chloride ($FeCl_3$), potassium ferricyanide solution $K_3Fe(CN)_6$, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), and sulfuric acid (H_2SO_4). All chemicals used were obtained from either Sigma-Aldrich (Madrid, Spain) or Merck (Darmstadt, Germany).

Plant sampling

Samples of the *R.raetam* twigs were collected in February 2019 during the flowering period in the region of Bousemghoun, El Bayadh-Algeria (Latitude: 32.8643, longitude: 0.02012 32°51′51″ North, 0°1′12″ East). The plant used was identified by the botanist Amar Eddoud (Department of Biology at the Kasdi Merbah University of Ouargla-Algeria). The twigs of the plant were dried in amber and then ground into a fine powder for later use.

Preparation of plant extracts

The extraction of secondary metabolites was performed using five different extraction techniques including maceration, reflux, Soxhlet, MAE, and UAE. The extraction yields were calculated using the following formula (1):

$$\text{Yield of extract [\%]} = \frac{\text{weight of extracts from plant sample [g]}}{\text{weight of dried plant sample [g]}} \cdot 100\% \qquad (1)$$

Conventional extraction techniques

The maceration method was performed by continuously stirring the mixture of plant powder (10 g) with 100 mL of solvent (water or ethanol 70%) at room temperature for 24 hours in the dark. The same solvent/sample ratio was boiled at 100°C in a 100 mL water bath reflux system for 15 min for heat reflux and Soxhlet extraction. The obtained mixtures were filtered with N°1 Whatman, and centrifuged at 3000 rpm for 5 min. The extracts were then delipidated with a petroleum ether solvent and evaporated to dryness in a rotary vacuum evaporator. The resulting residues were stored at 4°C until use.

MAE and UAE process

The MAE was carried out according to the protocol described by (Olalere and Gan 2021). 10 g of plant powder was added to 100 mL of different solvents (water, ethanol 70%). The mixture was irradiated for 10 min in a discontinuous process using a model microwave apparatus that operates at a frequency of 2450 kHz. The microwave equipment has been modified to condense the vapors generated during extraction. Concerning the UAE, it was carried out according to the method described (Nguyen Tram Anh and Van Hung et al. 2021), by using an ultrasonic bath at a frequency of 35 kHz, power of 20 W.

The obtained mixtures were filtered with N°1 Whatman and centrifuged at 3000 rpm for 5 min. The extracts were then delipidated with a petroleum ether solvent and evaporated to dryness in a rotary vacuum evaporator. The resulting residues were stored at 4°C until use.

Assessment of phytochemicals

The assessment of total polyphenol content (TPC) in different extracts was determined using the Folin-Ciocalteu spectrophotometric assay according to (Serairi-Beji and Wannes et al. 2018). TPC in the sample was calculated as milligrams of gallic acid equivalents per gram of dry residue (mg GAE/g DR). The evaluation of total flavonoid content (TFC) was measured by using the $NaNO_2$-$Al(NO_3)_3$-NaOH system (Dewanto and Wu et al. 2002) with minor modifications. It was expressed as milligrams of (+)-catechin equivalent per gram of dry residue (mg CE/g DR). The estimation of total tannin content (TTC) was assayed using the method of (Serairi-Beji and Wannes et al. 2018) with minor modifications. It was expressed as milligrams (+)-of catechin equivalents per gram of dry residue (mg CE/g DR).

DPPH• scavenging activity

The method described by (SÁNCHEZ-MORENO and LARRAURI et al. 1998) was used to measure the DPPH• scavenging activity. 50 µL of each extract at different concentrations (from 0.078 to 5 mg/mL) were added to 1.95 mL of DPPH• methanolic solution (0.025 g/L). Simultaneously, a negative control was prepared by combining 50 µL of methanol with 1.95 mL of DPPH• methanolic solution. The mixture was briskly shaken before being allowed to stand at room temperature for 30 minutes in the dark, at 515 nm. The absorbance of the resulting solution was measured. The scavenging activity was represented as IC_{50} mg/mL, which is the dose necessary to inhibit DPPH• by 50%. The percentage of DPPH• scavenging was calculated according to the following equation (2):

$$\text{DPPH} \bullet \text{ scavenging } [\%] = \frac{(\text{ABS0} - \text{ABS1})}{\text{ABS0}} \cdot 100 \quad (1)$$

ABS0 = absorption of control negative (DPPH solution without extract).
ABS1 = absorption of the sample.

Ferric reducing antioxidant power assay (FRAP)

The FRAP is determined according to the method described by (WU and SUN et al. 2014). 1 mL of the extract was combined with 2.5 mL of a 0.2 M phosphate buffer solution (pH 6.6) and 2.5 mL of a potassium ferricyanide solution $K_3Fe(CN)_6$. For 20 minutes, the mixture is incubated in a water bath at 50°C. After that, 2.5 mL of 10% trichloroacetic acid is added to halt the reaction, and the tubes are centrifuged for 10 minutes at 3000 rpm. A portion of the supernatant (2.5 mL) is mixed with 2.5 mL of distilled water and 0.5 mL of an aqueous 0.1 percent $FeCl_3$ solution. The absorbance of the reaction medium is measured at 700 nm against a similarly prepared blank, with the extract replaced by solvent, allowing the device to be calibrated (UV-VIS spectrophotometer).

Statistical Analysis

In the present study, all the trials were performed three times and their results were expressed as mean ± Standard Error of the mean and analyzed using the Sigma-Plot version 11.0 program. Statistical analyses were performed by analysis of variance ANOVA (Anova One way), followed by Tukey's test. The difference was considered statistically significant when $p < 0.05$ compared to the negative control. IC_{50} and EC_{50} values are

calculated by a basic and simple method based on nonlinear modeling between X (concentrations) and Y (response) using Origin Pro program version 2016.

Results and Discussion

Phytochemical assessment

Green chemistry is the need of the hour and the light of the future, revealing important information for science-based environmental conservation (ASIF and IMRAN 2021). To the best of our knowledge, no previous research has been conducted using GC methods to extract secondary metabolites from *R. raetam* twigs. For this reason, the purpose of this study was to compare the effectiveness of MAE and UAE methods as an innovative and eco-friendly technology to conventional extraction techniques including maceration, reflux, and Soxhlet.

The different extracts obtained were evaluated for their levels of yield, TPC, TFC and TTC. The results of this evaluation are summarized in Table 1.

Table 1

Yield%, TPC, TFT, and TTC of crude aqueous and ethanol extracts of *R. raetam* twigs

Method	Extraction yield [%]		TPC [mg GAE/g DR]		TFT [mg CE/g DR]		TTC [mg CE/g DR]	
	H_2O	EtOH 70%	H_2O	EtOH 70%	H_2O	EtOH 70%	H_2O	EtOH 70%
Maceration	18.89^{a}	14.68^{c}	155.13 ± 1.7^{c}	106.51 ± 1.89^{d}	8.69 ± 0.88^{d}	31.51 ± 1.28^{c}	9.68 ± 1.87^{b}	15.17 ± 2.22^{a}
Reflux	13.10^{c}	9.80^{e}	120.77 ± 2.5^{d}	136.05 ± 1.16^{b}	11.25 ± 1.17^{d}	25.61 ± 1.17^{c}	2.80 ± 1.87^{e}	5.17 ± 1.55^{d}
Soxhlet	11.64^{a}	12.40^{d}	142.6 ± 2.76^{b}	136.17 ± 1.89^{b}	9.20 ± 1.55^{e}	22.79 ± 2.46^{d}	$6,15 \pm 1.53^{c}$	10.27 ± 2.04^{b}
M A E	14.71^{b}	20.64^{a}	175.71 ± 2.09^{a}	160.43 ± 1.42^{a}	12.28 ± 0.92^{b}	39.97 ± 1.12^{a}	13.41 ± 3.05^{a}	14.78 ± 2.72^{a}
U A E	12.87^{c}	16.45^{b}	146.17 ± 2.2^{b}	111.91 ± 1.49^{c}	15.61 ± 1.93^{a}	32.28 ± 1.56^{b}	4.98 ± 2.04^{d}	6.94 ± 1.22^{c}

Explanations: Results are shown as mean ± Standard Error of the Mean (SEM). Comparison between groups was made using Tukey's test. Columns not sharing a common letter (*a–e*) differed significantly at $p < 0.05$

According to the reported findings, the highest yield obtained using water as solvent was 18.89% for maceration. following in decreasing order, by MAE (14.71%), reflux (13.17%), UAE (12.87%) and lastly the Soxhlet (11.64%). While, as for the hydro-ethanol mixture, the highest yield was obtained by the MAE method (20.64%) followed by UAE (16.45%), maceration (14.68%), Soxhlet (12.4%) and lastly reflux (9.8%). According to these results, the maceration technique yielded the best yield when using water as a solvent. The MAE process yielded the best yield when utilizing

hydro-ethanolic solution as a solvent. In some previous studies, extraction yields for *R.raetam* twigs were reported to be between 15% and 20%, using maceration of the aerial part of the plant in water and methanol (CONFORTI and STATTI et al. 2004, ALGHAZEER and EL-SALTANI et al. 2012, DJEDDI and KARIOTI et al. 2013).

The significantly higher TPC was recorded for the aqueous extracts ranging from 106.51 ±1.89 mg GAE/g DR to 175.71 ±2.09 mg GAE/g DR for maceration and MAE, respectively. The use of hydro-ethanolic solvent resulted in the highest TPC for MAE with a value of 175.71 ±2.09. These results are in agreement with those of (MARIEM and HANEN et al. 2014) as well as (ALGHAZEER and EL-SALTANI et al. 2012). Where their reported values were 137 and 89.35 ±2.1 mg GAE/g DR, respectively. However, our results are higher than those reported in the study of (SAADA and FALLEH et al. 2018) (27.75 ±0.02 mg GAE/g DR) and (DJEDDI and KARIOTI et al. 2013) (25.19 mg GAE/g DR). In their studies, they used conventional extraction methods such as maceration and Soxhlet for the aerial part of *R. raetam* using water and hydro-methanolic solvent, respectively.

The assessment of TFC revealed that the two non-conventional extraction methods (MAE and EAU) recorded the highest levels with values of 12.28 ±0.92 and 15.61 ±1.93 mg EC/g DR for the aqueous extracts, respectively, and 39.97 ±1.12 and 32.28 ±1.56 mg EC/g DR for the hydro-ethanolic extract, respectively. In comparison, (MARIEM and HANEN et al. 2014) found a TFC of 5.1 mg EC/g DR for the aqueous extract prepared by maceration of the aerial part of the same species. Whereas the TTC estimation indicated that MAE and maceration yielded the best extraction contents with values ranging from 13.41 ±3.05, 9.86 ±1.87 mg EC/g DR for aqueous extracts and 14.78 ±2.72, 15.17 ±2.22 mg EC/g DR for hydro-ethanolic extracts, respectively, for MAE and maceration. The MAE and maceration methods gave the best extraction contents for the two solvents. The results of TTC were not significantly different from those of the study carried out by (MARIEM and HANEN et al. 2014), who reported a content of 10.43 (mg EC/g DR) for the aqueous extract prepared by maceration.

The statistical study showed a highly significant difference ($P < 0.001$) for the different methods employed, solvents and method-solvent interaction. It can be deduced that the nature of the solvent and the extraction method significantly influence the contents of phytochemicals and their antioxidant abilities. This result is in agreement with those reported by several authors (DAHMOUNE and BOULEKBACHE et al. 2013, MANSOURI and LOVILLO et al. 2021). These results are consistent with the findings of (ZAOUI and OUGHLISSI-DEHAK et al. 2021), where the authors indicated

that the MAE process of *Calycotome spinosa* allowed obtaining a greater yield when hydro-ethanol was used instead of conventional methods.

This observation could be explained by the important dipole moment of both solvents (Boeing and Barizão et al. 2014) as well as their important dielectric constant (Dahmoune and Boulekbache et al. 2013). In this context (Cavalloro and Martino et al. 2021) reported that the use of the mixture (water-organic solvent) can facilitate the extraction of bioactive substances that are soluble in water and/or in the organic solvent. The effectiveness of MAE is certainly due to its mode of action, which is completely different from conventional methods. Microwave irradiation causes a more efficient perturbation of the cell structures, leading to the rupture of the cell wall and membranes. In addition, in the case of a hydroalcoholic mixture combining two polar solvents, the mixture is heated very quickly, which increases its penetration into the matrix, thus facilitating the liberation of the cell contents optimally (Al Jitan and Alkhoori et al. 2018). The MAE technique is very easy to implement, very fast and requires less solvent than conventional methods. Consequently, it allows to avoid the degradation of thermolabile compounds (Delazar and Nahar et al. 2012, Gourguillon and Destandau et al. 2016). In the same context, the study conducted by (Liazid and Palma et al. 2007) showed that there is a relationship between the chemical structure and the stability of phenolic molecules during the MAE process. In the case of the UAE technique, it is important to note that the results obtained are not very different from those obtained by conventional methods. The low power and lack of reproducibility of ultrasound applied directly to the sample could be attenuated by the water in the ultrasonic bath and the glassware used for the experiment, as highlighted by (Chemat and Rombaut et al. 2017). Both the reflux and Soxhlet methods yielded rather lower levels, especially for flavonoids and tannins. The thermal degradation of these compounds during prolonged heating could be the main cause, as confirmed by (Sutar and Garai et al. 2010, Karami and Emam-Djomeh et al. 2015), and (Perva-Uzunalić and Škerget et al. 2006). Considering the last study, the degradation of catechins was observed at high extraction temperatures (95°C). For the maceration method, it showed moderate results, but its main disadvantage is that it requires several hours of extraction and large amounts of solvent. Furthermore, the study of (Roselló-Soto and Parniakov et al. 2016) on the application of non-conventional extraction methods for the sustainable and environmentally friendly production of valuable compounds from mushrooms showed that conventional extraction methods usually involve water or organic solvents and can lead to significant degradation of the constituents, and shows the great potential of

these environmentally friendly methods for the eco-friendly production of specific compounds to be used as nutraceuticals or as functional food ingredients.

Antioxidant Activities

DPPH• scavenging activity and FRAP were used to evaluate the antioxidant activity of the different *R. raetam* extracts. The results shown in Figure 1 and Table 2 indicated that the extracts from both solvents with MAE exhibited the highest significant DPPH• scavenging capacity (IC_{50} values of 0.455 ±0.075 mg/mL and 0.34 ±0.39 mg/mL, respectively).

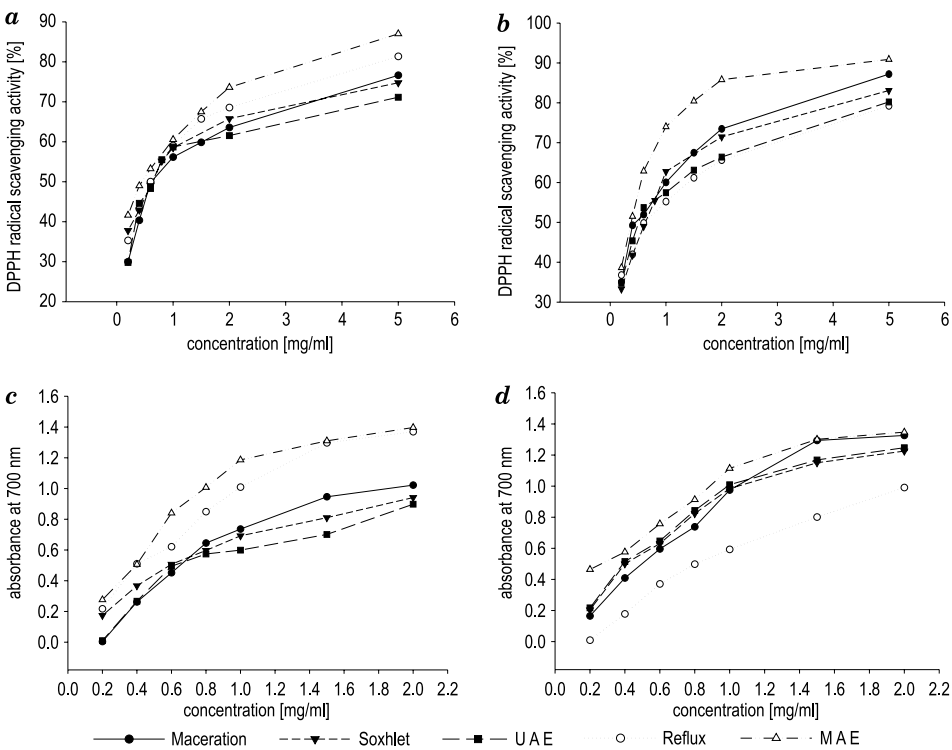

Fig. 1. Graph comparing the performances of the antioxidant activities of different sample extracts of *R. raetam* twigs: *a–b* – DPPH• scavenging activity; *c–d* – FRAP of aqueous and hydro-ethanolic extracts, respectively

In addition, FRAP results showed that the extract prepared by MAE using water and hydro-ethanolic solution had the highest efficiency for reduction (EC_{50} of 0.35 ±0.022 and 0.28± 0.016, respectively). These results are in agreement with the study of (SAADA and FALLEH et al. 2018) (IC_{50} = 0.160 ±0.01 mg/mL) and (HAYET and MAHA et al. 2008)

(IC_{50} = 0.450 mg/mL). Moreover, another study conducted on other species of the genus *Retama* by (BELMOKHTAR and HARCHE 2014) showed that it was significantly able to quench the DPPH• (IC_{50} = 0.15 mg/mL). However, our results were lower than those previously reported by (MARIEM and HANEN et al. 2014) (IC_{50} = 0.043 mg/mL) for the aqueous extract of the aerial part.

Table 2
DPPH• scavenging activity and FRAP of crude aqueous and ethanol extracts of *R. raetam* twigs

Method	IC_{50} for DPPH• test		EC_{50} for FRAP• test	
	H_2O	EtOH 70%	H_2O	EtOH 70%
Maceration	0.72 ±0.04^c	0.52 ±0.04^b	0.61 ±0.03^d	0.49 ±0.04^d
Reflux	0.59 ±0.05^b	0.68 ±0.07^c	0.43 ±0.05^b	0.43 ±0.05^c
Soxhlet	0.70 ±0.06^c	0.55 ±0.060^b	0.57 ±0.03^c	0.35 ±0.03^b
M A E	0.45 ±0.07^a	0.34 ±0.03^a	0.35 ±0.02^a	0.28 ±0.01^a
U A E	0.65 ±0.06^b	0.57 ±0.04^b	0.65 ±0.03^e	0.42 ±0.01^c

Explanations: Results are shown as mean ± Standard Error of the Mean (SEM). Comparison between groups was made using Tukey's test. Columns not sharing a common letter (*a*–*e*) differed significantly at $p < 0.05$

Correlation study

The results presented in Figure 2 show a significant negative correlation between the TPC and the IC_{50} values (R^2 = -0.91) and also with the EC_{50} (R^2 = -0.84). On the other hand, the IC_{50} and EC_{50} values are substantially correlated with an R^2 of about 0.85. The correlation coefficients (R^2 = -0.62, -0.35, -0.72 and -0.57) show that the flavonoid and tannin content of the extracts have a poor relationship with the antioxidant activity. The high values of R^2 obtained in the correlation study shows that polyphenol content and antioxidant activity are strongly correlated. These results are in agreement with many authors (HOSU and CRISTEA et al. 2014, ANJUM and TRIPATHI 2020, KAINAMA and FATMAWATI et al. 2020). It can be concluded that the TPC present in the extracts is mainly responsible for their antioxidant activity. Several authors have suggested that the polar molecules present in plant extracts contribute considerably to increasing their antioxidant activity (CASAGRANDE and ZANELA et al. 2018, NIROULA and AMGAIN et al. 2021). Its activity is due to their ability to release hydrogen (CHIORCEA-PAQUIM and ENACHE et al. 2020).

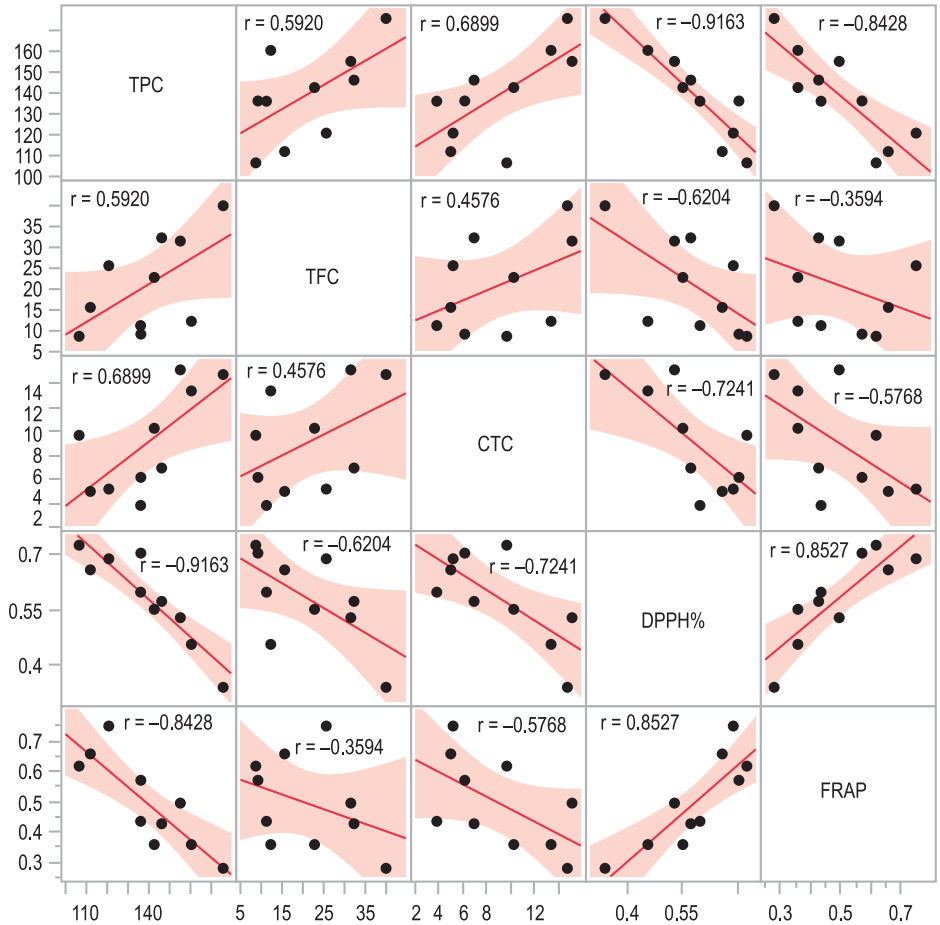

Fig. 2. Correlation matrix between TPC, TFC, TTC, DPPH• scavenging%, and FRAP of different sample extracts of *R. raetam* twigs

Conclusion

The findings of the present investigation indicate that MAE proved to be the most efficient technique yielding the highest levels of phytochemicals obtained from *R. raetam* twigs and exhibiting highly significant antioxidant activity, suggesting that it could be a good source of phytopharmaceutical molecules such as natural antioxidant drugs. Conventional techniques showed much less efficiency as compared to both modern techniques (MAE and UAE).

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