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CENTRAL COMPOSITE DESIGN (CDD) FOR THE OPTIMIZATION OF TOTAL FLAVONOIDS EXTRACTION FROM *TRICHILIA EMETICA* ROOT BARK UNDER ECO-FRIENDLY CONDITIONS AND ANTIOXIDANT ASSESSMENT

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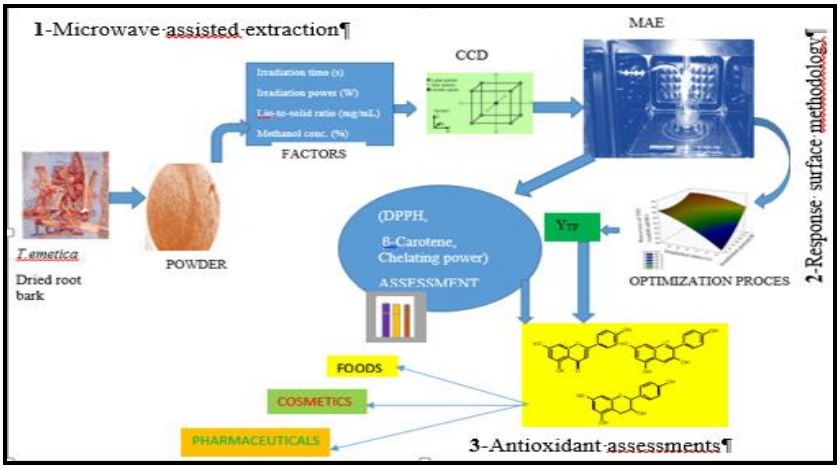
ABSTRACT

A seek was made in this study to determine the influence of different factors on the extraction process of *Trichilia emetica* (Vahl) root bark. Conventional solvent extraction (CSE) and microwave assisted extraction (MAE) were practised, then compared. The obtained extracts were used to conduct different antioxidant investigations. A monothetic analysis method was used for the designing experiments. Four independent variables were tested and the maximum extraction efficiency was achieved with irradiation time X1 of 90 s, microwave irradiation power X2 of 700 W, liquid-to-solid ratio X3 of: 20:0.7 (mL/g) and methanol concentration X3 30%. The total flavonoids content of 529.01 µg QE/g DW were obtained under these optimum conditions. The antioxidant investigation of the later MAE extract exhibited the highest values of DPPH radical-scavenging assay with inhibition percentage (IP) of 86 %, an antioxidant activity (AOA) of 90.36 % in the *β*-carotene bleaching test and the chelating power (CP) of 82.5% compared to CSE. The total flavonoids contents obtained by microwave-assisted extraction were twice those obtained by conventional solvent extraction. Second-order polynomial model could be employed to obtain the maximum total flavonoids. A correlation study of a mathematical regression model and "one-variable-at-a-time" (OVAT) were used for describing the effects of the MAE factors on the extraction. The response surface methodology is conductive to optimize the extraction process. With such good antioxidant potentials of flavonoids from *T. emetica* root bark, this plant is suitable to solve the antioxidant deficiency manifested over time by the body through degenerative diseases and oxidative stress.

KEYWORDS: *Trichilia emetica*, "one-variable-at-a-time", chelating power, DPPH$^{\bullet}$, *β*-carotene assay, flavonoids.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The presence of a number of hydroxyl group on the flavonoid skeleton, justified the several biological activities attributed to the flavonoids. In addition to the main antioxidant activity, we frequently encounter flavonoids with antihypertensive, neuroprotective,

cardioprotective, anti-inflammatory, and antiviral properties, hence their countless virtues in the field of health (Yi Cao *et al.,* 2021). Nowadays, the growing interest in flavonoids make that more attention was paid to the search for novel plant with phenolics compounds, and specifically with flavonoids. (Xu-Yang et *al*, 2021). Flavonoids from medicinal plants have an undeniable interest due to their numerous health benefits (Scalbert *et al.,* 2005; Rasouli *et al.,* 2017; Taamalli *et al.,* 2019). Those biological activities being linked to the active ingredients, depend upon the type of flavonoids, the extraction process but also the type of extraction method. The extraction procedure, knowing as the first step for the selective removal of component in a plant material, guarantees the quality of the secondary metabolite and its active ingredient. However, to our knowledge, there is no standard extraction method for obtaining secondary metabolites-rich extracts; thus, it is dependent on applications of interest, selectivity, and the nature of the plant material cell (Shanmuganathan *et al.,* 2022). Microwave assisted extraction (MAE) has been used to increase biological activities, to reduce time, extraction solvent, and energy used compared to the conventional method (Nana et *al.,* 2021). There are a number of methods that can be used for extraction such as maceration, reflux extraction, Soxhlet extraction and supercritical fluid extraction called conventional method. However, these methods are either time, energy and solvent-consuming or too expensive for small-scale implementation. Therefore, microwave assisted extraction been have proposed to solve these shortcomings (Chumnanpaisont *et al*., 2014). This alternative extraction methods, has many advantages, including a strong penetrating force, high quality of the targeteted compounds. Hence many reports have been published on the application of Microwave assisted extraction of secondary metabolites from plants (Xu-Yan *et al.,* 2021). A plant's bioactivities are caused by the presence of bioactive secondary metabolites (Jain *et al*., 2019; Bindurani *et al.,* 2019).

T. emetica is a plant of the Meliaceae family, used in traditional African Pharmacopoeia for its many biological properties such as antioxidant, antiplasmodial, anti-inflammatory, antibacterial, antifungal properties (Dieng *et al.,* 2017). *Trichilia* comprises 90 species distributed in tropical America, continental Africa and madagascar, and it is widely use as antioxidant, antimalarial, and antipyretics in traditional medecine (Nnabuk *et al.,* 2016). Previous Sanogo studies showed the hepatoprotective activities of *T. emetica* against CCl4-induced liver injury *in vivo* (Sanogo *et al.,* 2001). The antioxidant properties of plants are generally attributed to their content of phenolic compounds, particularly flavonoids and tannins (Santos-Sanchez *et al.,* 2014; Pavun *et al.,* 2018). The antioxidants, beyond the enhancement of our immune system, are primordial for the good health of our body. More than prevent many pathologies, they have an effect against free radicals and prevent premature aging. Plants are also a source for obtaining natural antioxidants as living organism. For example, the most powerful antioxidant astaxantin, a natural red pigmented ketocarotenoid from plant-like protist is extracted from living organism *Haematococcus pluvialis*. In order to increase the active ingredient in the extraction, and thus improve the biological activity, the optimisation of the extraction technique is necessary. Hence the interest of this work namely the optimization of the microwave-assisted extraction of total flavonoids from the root bark of *T. emetica* and the antioxidant assessment. This optimization will provide mathematical models able to properly predict the behaviour of the system considering the factors that influence the MAE process. For the best of our knowledge, no study is reported on the optimisation of microwave-assisted extraction of flavonoids from species *Trichilia emetica* root bark. Therefore, the objectives of this study are as follows

(1) To analyze the extraction characteristics of flavonoids from *T.emetica* under microwave conditions in terms of irradiation time, irradiation power, methanol concentration, and solid-to-liquid ratio.

(2) To determine the optimum parameters for the highest flavonoids extraction yield and the recovery total flavonoids which occur the best antioxidant percentage.

2. METHODS

2.1. Plant material

The root bark of *Trichilia emetica* was harvested in December 2022, from a forest in a suburb around Ngaoundere (Adamawa Region of Cameroon). The plant sample was taxonomically authenticated by Dr Fawa, a botanist, the head of the Department of Sciences and Technology of Organic Agriculture (STOA), the Faculty of Science of the University of Ngaoundere. The plant material was freed of extraneous material, shade-dried at room temperature for two weeks and milled to a fine powder, using a waring blender. Three kilograms of powder was packaged in an air tight container, labelled and stored until used.

2.2 Chemicals

As Chemicals, and materials, Ferrozine, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, sodium carbonate, potassium acetate, potassium hexacyanoferrate, hydrated iron (II) chloride, iron (III) chloride, β-carotene, linoleic acid, chloroform, tween 40, methanol, and ferrozine, obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Metertech spectophotometer UV/Vis sp 8001, rotary evaporator system (Büchi, Switzerland) and FCMCR-3C-W type Microwave Chemical Reactor **(**Gongyi City Kerui Instrument Co., Ltd**)** were those used in this work.

2.3 Microwave-Assisted Extraction technique

This eco-friendly extraction method of flavonoids were performed with a FCMCR-3C-W type Microwave Chemical Reactor **(**Gongyi City Kerui Instrument Co., Ltd). The apparatus operated at a frequency of 2450 MHz ± 50Hz and a maximum output power of 900W

with a continuous wave as output mode**.** Precisely, the vessel was introduced into the microwave cavity and fitted with a condenser. For the microwave extraction operation, 1 g of dried powder of vegetal material was suspended in 20 mL of aqueous methanolic solution in a 150 mL Teflon extraction vessel. The microwave extraction was carried out at different irradiation power (%) over different lengths of irradiation time (s.) in different solvent concentrations (% v/v). After the extraction, the vessel was allowed to cool down (1 min at 25°C), and the mixture filtered using Whatman filter paper. The filtrate was kept at 4°C for the different uses.

Fig. 1: Microwave Chemical Reactor FCMCR-3C-W (Gongyi City Kerui Instrument Co., Ltd).

2.4 Experimental design for the optimisation of MAE of the recovery of total flavonoids

The central composite design via a response surface methodology was used for the optimization of the microwave assisted extraction conditions in order to recover maximum total flavonoids content. Base on the "one-variable-at-a-time" (OVAT), the impact of change in one factor is investigated on the total flavonoids content when all the three other factors are kept constant. Table 1 below shown the experimental matrix for lower and higher values for each of the factors. The experimental variables and the twenty eight experimental points is presented in table 2 from where we can see the non-coded values. The central composite design consists of twenty eight experiments were conducted in triplicate in order to minimize the effects of unforeseen variability on the total flavonoids content. The DPPH radical-scavenging assay, the *β*-carotene bleaching test and the chelating power (%) were calculated according to their different formula. Response surface methodology is a multi-response optimization technique when the interest responses are affected by multiple variables named factors (Mongomery, 2017). Then, the used of response surface methodology (RSM) for optimization of flavonoid extraction conditions has high accuracy. The linear, interaction and quadratique effects of factors during microwave assisted extraction can be determined using the central composite design (CCD) (Azahar *et al.*, 2017).

Table 1: **Matrix of coded variables of the central composite design of MAE.**

Run	Coded values				Actual values				Response
	X1	X2	X3	X4	Time (s)	Power (W)	Ratio (mLg^{-1})	Conc. (%)	
1	-1	-1	-1	-1					Y1
2	1	1-	-1	-1					Y2
3	-1	1	-1	-1					Y3
4	1	1	-1	-1					Y4
5	-1	-1	1	-1					Y5
6	1	-1	1	-1					Y6
7	-1	1	1	-1					Y7
8	1	1	1	-1					Y8
9	-1	-1	-1	1					Y9
10	1	-1	-1	1					Y10
11	-1	1	-1	1					Y11
12	1	1	-1	1					Y12
13	-1	-1	1	1					Y13
14	1	-1	1	1					Y14
15	-1	1	1	1					Y15
16	1	1	1	1					Y16
17	-1.60717	0	0	0					Y17
18	1.60717	0	0	0					Y18
19	0	-1.60717	0	0					Y19
20	0	1.60717	0	0					Y20
21	0	0	-1.60717	0					Y21
22	0	0	1.60717	0					Y22
23	0	0	0	-1.60717					Y23
24	0	0	0	1.60717					Y24
25	0	0	0	0					Y25
26	0	0	0	0					Y26
27	0	0	0	0					Y27
28	0	0	0	0					Y28

Table 2: Coded and undcoded levels design for different factors.

	Coded levels design for optimisation				Uncoded values for independent variables			
Run	**X1**	**X2**	**X3**	**X4**	**X1 (s)**	**X2 (W)**	**X3 (g/mL)**	**X4 (%)**
1	-1	-1	-1	-1	70	500	2.77:20	30
2	1	-1	-1	-1	90	500	2.77:20	30
3	-1	1	-1	-1	70	700	2.77:20	30
4	1	1	-1	-1	90	700	2.77:20	30
5	-1	-1	1	-1	70	500	3.22:20	30
6	1	-1	1	-1	90	500	3.22:20	30
7	-1	1	1	-1	70	700	3.22:20	30
8	1	1	1	-1	90	700	3.22:20	30
9	-1	-1	-1	1	70	500	2.77:20	50
10	1	-1	-1	1	90	500	2.77:20	50
11	-1	1	-1	1	70	700	2.77:20	50
12	1	1	-1	1	90	700	2.77:20	50
13	-1	-1	1	1	70	500	3.22:20	50
14	1	-1	1	1	90	500	3.22:20	50
15	-1	1	1	1	70	700	3.22:20	50
16	1	1	1	1	90	700	3.22:20	50
17	-1.60717	-1	-1	-1	63.938	500	2.77:20	30
18	1.60717	-1	-1	-1	96.071	500	2.77:20	30
19	0	-1.60717	-1	-1	80	439.283	2.77:20	30
20	0	1.60717	-1	-1	80	760.717	2.77:20	30
21	0	0	-1.60171	-1	80	600	2.636:20	30
22	0	0	1.60717	-1	80	600	3.353:20	30
23	0	0	0	-1.60717	80	600	2.99:20	23.928
24	0	0	0	1.60717	80	600	2.99:20	56.071
25	0	0	0	0	80	600	2.99:20	40
26	0	0	0	0	80	600	2.99:20	40
27	0	0	0	0	80	600	2.99:20	40
28	0	0	0	0	80	600	2.99:20	40

X_1: Irradiation time (s), X_2: Microwave power (W), X_3: Liquid-to-solid ratio (mL/g) and X_4: Methanol concentration (%)

2.5 Analysis of chemical and antioxidant activity

Any secondary metabolites present at low concentrations levels compared with those of an oxidizable substrate, significantly slows down or prevents oxidation of substrate are and antioxidant compounds (Krinsky, 1992). The synthetic antioxidants are not without drawbacks, notably liver damage carcinogenic and toxic effects… low water solubility, hence need for the natural exogenous antioxidants plants with their free radical scavenging activity (polyphenols, alkaloids and terpenoids) is need. More, the efficiency of frequently used exogenous antioxidants appears most probably from the increase of the endogenous free radical scavengers as enzymes in human body (Nabilah et *al*., 2011).

2.5.1 Quantitative estimation of total flavonoid content

The evaluation of the total flavonoid content was carried out by the method used by Zhishen *et al.* (1999). Precisely, 50 mg of each fraction was dissolved in 10 mL of 80 % aqueous methanol and filtered through Whatman filter paper number 1 (40 mm). In a 10 mL test tube, 300 µL of each extract, 3.4 mL of 30% methanol, 150 µL of 0.5 M $NaNO_2$, and 150 µL of 0.3 M $AlCl_3{\cdot}6H_2O$ were added and mixed, followed by 5 min incubation and the addition of 1 mL of NaOH (1 M). Absorbance was measured at 510 nm with a spectrophotometer. The standard curve for total flavonoids was made using Quercetin standard solution (0-100 mg/L) using the same aforementioned procedure. The total flavonoid content was shown as milligram of Quercetin equivalents (QE) per gram dry matter of extract.

2.5.2 Quantitative antioxidant test using the Chelating Power on Fe2+ Ions

By using chelating power method for antioxidant evaluation, the potentiality of the extract to chelate iron (II) was investigated based on the method of Dinis *et al.*, (1994) with slight modifications. Various sample solutions (50–300 µg/mL) were prepared with dissolving the extracts in the methanol. An aliquot of each sample (200 µL) was mixed with 100 µL of $FeCl_2{\cdot}2H_2O$ (2 mM) and 900 µL of methanol. After 5 min incubation, an initial reaction was fuelled by the addition of 400 µL of ferrozine (5 mM). After 10min incubation, the absorbance at 562 nm was recorded. The percentage of the chelating activity was calculated based on the following equation:

Chelating activity (%) = $\frac{(\text{absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \times 100$

2.5.3 Quantitative antioxidant test using DPPH radical-scavenging activity

The scavenging effect of the extracted flavonoids on the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined by the method described by Ge *et al.*, (2020) with some modifications. The evaluation of these activities is described as follow: The solution of DPPH was prepared by dissolving 2 mg in 50 ml of methanol. The fractions were added to DPPH so as to have 1 ml of solution of 0.5000, 0.2500, 0.1250, 0.1000, 0.0500, 0.0250, 0.0125 ml/mg of concentration in the spectrophotometric curves. These curves were introduced in the spectrophotometer and the optical densities read at 517 nm after 30 min of incubation. The negative witness is a solution of DPPH at 10 % in the methanol and the positive witness is the butylhydroxytoluene (BHT) were submitted to the same analysis and rigorously in the same conditions with the same concentration as the plant fractions. Inhibition of free radical by DDPH in percent (I %) was calculated by using the formula below.

$$I\% = \frac{(A\ \text{blank} - A\ \text{sample})}{A\ \text{blank}} \times 100$$

Where A blank is the absorbance of the control reaction and A sample is the absorbance of the test compound. The optical densities obtained helped to draw the graphic according to the inhibition concentrations and the concentration in inhibitors and to deduct the IC_{50} values.

2.5.4 Quantitative antioxidant evaluation using β-carotene bleaching test

The estimation of the β -carotene concentration was achieved by measuring the inhibition of the oxidative degradation of the β –carotene occur by discoloration due to the oxidation products of linoleic acid. According to the method described by Kartal et *al* (2007). With slight modification, the emulsion of beta carotene/ linoleic acid couple is obtained by solubilizing 0.25 mg of β -carotene in 0.5 ml of chloroform, 15 µL of linoleic acid and 100 mg of tween 40 are added; the chloroform is completely evaporated using a vacuum evaporator. Subsequently 50ml of distilled water saturated with oxygen are added, the resulting emulsion is vigorously shaking to form an emulsion. 350 µL of solution of extracts or reference BHT dissolved in methanol (2mg/ml) are added to 2.5ml of previous emulsion. The kinetics of discoloration of the emulsion in the presence or and absence of antioxidant (negative control in which sample is replaced by methanol) is followed at 490nm using Metertech Germany spectrophotometer UV/Vis sp 8001. The test tubes were incubated at 50°C in a bath water for two hours. A blank treatment served as the control for the Spectrophotometric readings. Antioxidant activity (AOA) was calculated using the following equation.

$$\%AOA = \frac{A2H}{Ai} \times 100;\ \text{with}\ \%$$

AOA, antioxidant activity; A2H, β-carotene content after 2 h of assay; Ai, initial β-carotene content

2.6 Statistical analysis

The results reported in this work are the average values of three measurements. Tests of statistical significance (ANOVA and Fisher's least significant difference) were carried out using Stat graphics plus XVI software and the sigma plot 12.0 software was used for the graphs. The linear coefficients, quadratic, and interaction were determined by least squares regression, followed by analysis of variance (ANOVA). Values of P <0.05 were considered significant.

The analysis of variance (ANOVA) was also used to determine the influence of each factor and the degree of significance of each of these effects. It then examines the statistical significance of each effect by comparing the squared average against an evaluation of the experimental error. The flavonoids with different antioxidants assays and its significance was tested using Student's t-test (<0.05).

3 RESULTS AND DISCUSSION

An efficient and efficacy extraction methods prove to be a crucial step for getting extracts with high yields and strong antioxidant activity (Esmaeili *et al.*, 2015). Optimization of microwave assisted extraction makes it possible to obtain maximal yield under optimal conditions.

3.1 Total flavonoid content from the two extraction methods

It emerges from this study that, the total flavonoids content from microwave-assisted extraction with methanolic aqueous solution, is two times the corresponding values obtained with conventional extraction with the same extraction solvent at the same temperature and for all the different extraction times. Table 3 below shows. This is mainly because microwave energy is delivered efficiently to materials through molecular interaction with the electromagnetic field and offers a rapid transfer of energy to the extraction solvent and raw plant materials (Zhang *et al*., 2008). In addition, using microwave energy to extract compounds from plant materials, by application of the adsorption kinetic equations to the solid–liquid extraction of total flavonoids from *T. emetica* root bark, the Peleg kinetic model is the one best adjust the extraction of total flavonoids (Nana *et al.*, 2023).

Table 3: **Microwave-assisted and conventional extraction of total flavonoids from *T. emetica* root bark**.

Extraction time (s)	Total flavonoids content (µg QE/g DW)	Ratio of MAE to Conventional

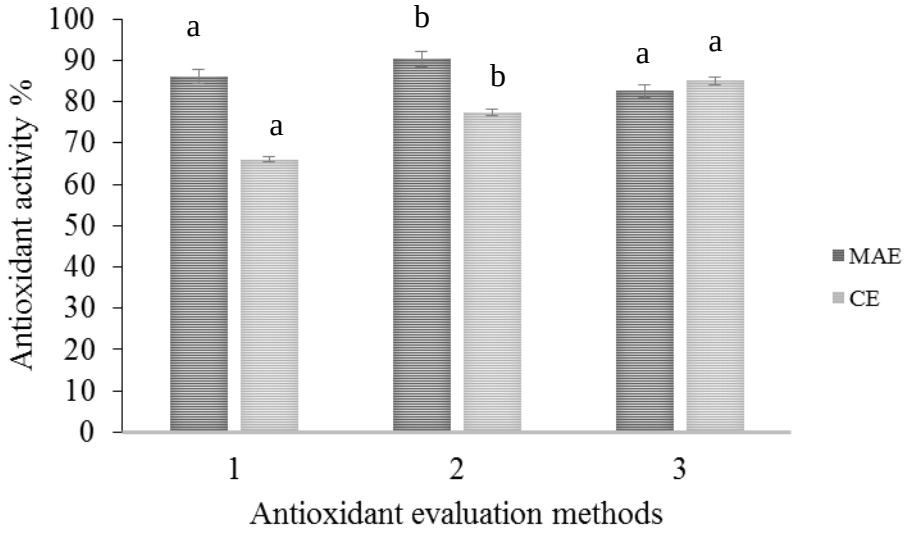
	MAE	CE	
10	107	43	2.48
20	112	52	2.15
30	120	55	2.18
40	119	54	2.20
50	123	62	1.98
60	120	59	2.03

MAE: Microwave Assisted Extraction, CSE: Conventional Solvent Extraction

In order to modelling the recovery of total flavonoids from *T. emetica* vegetal matrix, different models were used to fit the experiment data and evaluate the microwave-assisted extraction process. It emerges from this investigation that the Peleg kinetic model is the one best adjust the extraction of total flavonoids (Nana *et al.*, 2023).

3.2 Influence of the extraction methods on antioxidant capacity

The antioxidant capacities of the extracts were evaluated by DPPH radical assay, oxidative degradation of β-carotene assay and the iron chelating power assay. As shown in Fig. 2 below, MAE extract showed higher antioxidant activities for the two first methods as compared to CE however, for the iron chelation power the conventional solvent extraction showed a bit higher antioxidant activity than MAE. The effect of irradiation on the microwave extracts must have attenuated the sequestering effect of the Fe (II)- Ferrozin complex, thus influencing the chelating capacity of microwave-assisted extracts. This lower activity of MAE extract could result in high irradiation power thus preventing the chelating power. In addition, crude methanol extract was found to harbour better chelating capacity in conventional solvent extraction (Sudan *et al.*, 2014). Due to the presence of hydroxyl group, flavonoids and phenolic compounds can interact with transition metal ions to form chelates complexes, these complexes might be stable, or redox cycling might take place leading to the reduction of the iron or copper to a more pro-oxidant from the oxidized flavonoids (Rice-Evans, 2001). To prevent the human bodies from oxidation, flavonoids have chelating activity which allowed them to chelate or bind to metal ions (Nabilah et *al*., 2011).



1: Scavenging DPPH assay method; **2**: Oxidative degradation of β-carotene method; **3**: Fe (II) Chelation method

Fig. 2: Antioxidant activity of *T. emetica* roots bark extracts used different antioxidant assays. The bars with different letters for each antioxidant assay of extraction methods are significantly different (P < 0.05).

3.3 Influence of investigated factors on "*one variable-at-a- time*" design

This study aimed to enhance the recovery of total flavonoids from *T. emetica* root bark by optimizing the MAE condition and investigating the influential extraction factors. For this, the effects of different factors, such as solvent concentration, solvent-to-solid ratio, microwave power, and microwave irradiation time on the extraction yield were investigated. According to the irradiation time, it appears that the total flavonoids content increasing with the irradiation time up to 60 s, after that a decrease occurs. We can explain this increase value by the fact that the leaching phenomenon takes place immediately between the plant materials and the solvents leading to the dissolution of metabolites into the solution, the fall of the peak may be assigned to the

deterioration of metabolites with the increase in temperature of the reaction medium in more volatiles metabolites in prolonged heating time (Liu *et al.*, 2019). This revealed the nonlinear change of the total flavonoids content thus an optimal range from 70 to 90 seconds for irradiation time. Moreover, irradiation power, as irradiation time in the microwave assisted extraction process is a fundamental parameter. Hence to avoid an excessive temperature capable of destroying the metabolites, the choice of irradiation power must be made accordingly. We observe for optimal irradiation power a polynomial shape increasing up to 600 W. These results suggest a close link between extraction time and extraction power to consider for the optimization. It follows that for a high irradiation power a short extraction is necessary and likewise long exposition time, could be appropriate for short irradiation power values (Nana *et al.*, 2021). These observations reinforce the relevance of the study of the two previous parameters, their interactions for effective and efficient optimisation of the microwave extraction of flavonoids extracts from *T. emetica*. Liquid-to-solid ratio effects seen in Fig. 4C. Significant ($p < 0.05$) show the maximum TFC of 817 µg QE/100 g DW; this maximum was observed to be around the ratio $2\ 10^{-2}$– $4\ 10^{-2}$ mL/g. Numerous reports indicate that, the volume of solvent must be sufficient to allow total immersion of the plant raw material during the entire extraction process (Thomas-Michel., 2011). Results are within the range reported for the optimal liquid-to-solid ratio between (20: 0.4 to 20 : 0.8) (mL/g), with a pic to 20 : 0.6 (mL/g). The total flavonoid content ranges from 455 to 786 µg QE/gDW, with a pic of 817 µg QE/gDW. The increase observed can be explained by the principle of material transfer, the driving force during this transfer phenomenon is the concentration gradient between the solvent and the material which will be high when this ratio used is large. The decrease in content observed after the ratio of 20:0.6 (mL/g) is due to the saturation of the reaction medium leading to a low dissipation factor (tan δ) in the presence of microwaves. (Chen *et al*., 2007). Fig. 3D shows that the extraction of total flavonoids content was also affected by the aqueous methanol concentration. When the methanol concentration was lower than 40% (v/v), the extraction recovery of TFC was enhanced with the increase in methanol concentration. When the methanol concentration was up to 40% (v/v), the extraction slowly decreased with the further increased aqueous methanol concentration. The adjustment curve indicates that there is a decrease in TFC contents over time as the proportion of water decreases to pure methanol. Tan δ shows the ability of the solvent to convert microwave energy into thermal energy therefore, it was expected that a higher yield could be obtained using methanol due to its high ability to provide more heat into the extraction medium (Velisdeh *et al.*, 2021). This observation is logical because of the given dielectric constant of water (78.3) and that of methanol (32.6). Hence, choosing the appropriate solvent mixing system for MAE is fundamental to achieving an optimal extraction process. This factor choosing the best concentration thus conditions the solubility of the molecules of interest in the mixture. The solvent must be in accordance with the solubility of the active component. Generally, flavonoids in plants are water-soluble glycosides to be extracted with a hydroalcoholic solution (Badal *et al.*, 2017).

Extraction time: Whatever the extraction process, time remains a determining factor in the recovery of secondary metabolites. In the context of this work, the irradiation time actually influences the extraction microwave process. It's significantly ($P < 0.05$) influenced the total flavonoids. Hence, the recovery of TFC is affected by different extraction times as shown in Fig. 3A. The TFC content increased with the increased MAE time up to a maximum at a time after 80 s, before a decrease was observed.

Irradiation power: The power of the device influences the efficiency of extraction over time. The resulting phenomenon is that, irradiation power influences the rupture of plant cell membranes thus improving the penetration of the solvent. This parameter in the microwave assisted extraction process is also too important. The effect of microwave power in the OVAT design is presented in Fig. 3B. A polynomial shape increasing with increased irradiation power up to 600 W.

Liquid-to-solid ratio: The results of total flavonoids content recovery according to liquid-to-solid ratios can be seen in Fig. 3C. Significant ($p < 0.05$) effect of the liquid-to-solid ratio was observed with the maximum TFC of 817 µg QE/100 g DW; this maximum was observed to be around the ratio $2\ 10^{-2}$– $4\ 10^{-2}$ mL/g. Our results are within the range reported for the optimal liquid-to-solid ratio between (20:0.4 to 20:0.8) (mL/g), with a pic to 20:0.6 (mL/g). The total flavonoid content ranges from 455 to 786 µg QE/gDW, with a pic of 817 µg QE/gDW.

Methanol concentration: The nature of solvent is also a crucial parameter for a good extraction process. Thus Fig. 3D shows that the extraction of TFC was greatly influenced by the methanol concentration in water. When the methanol concentration was lower than 40% (v/v), the extraction recovery of TFC was enhanced with the increase in methanol concentration. When the methanol concentration was higher than 40% (v/v), the extraction slowly decreased with the further increased methanol concentration. The adjustment curve indicates that there is a decrease in TFC contents over time as the proportion of water decreases to pure methanol.

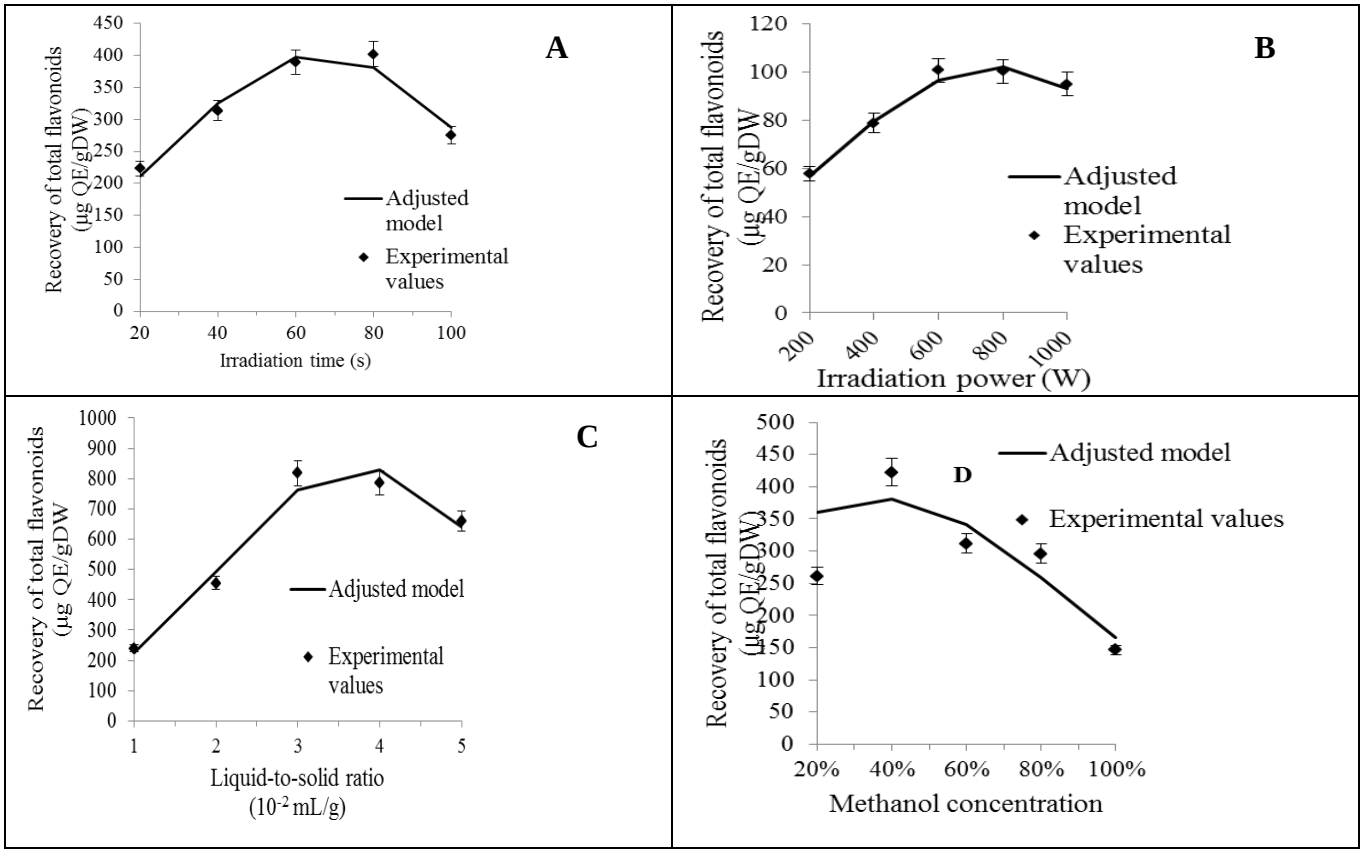


Fig 3: A Effect of irradiation time on the extraction of TFC (X2: 600W, X3: 20:1 mL/g X4: 95%).
B Effect of irradiation power on the extraction of TFC (X1: 60 s, X3: 20:1 mL/g, X4: 95%).
C Effect of liquid-to-solid ratio on the extraction of TFC (X1: 60 s, X2: 600W, X4: 95%).
D Effect of methanol concentration on the extraction of TFC (X1: 60 s, X2 600W, % X3: 20:1 mL/g).

3.4 Optimization of microwave-assisted extraction (MAE) process

The analysis by the centered composite design (CCD) method of 28 experiments carried out under the influence of the factors; irradiation time (X1), irradiation power (X2), liquid-to-solid (X3), and the methanol concentration (X4) on the extraction of total flavonoids by the microwave-assisted extraction method is made. During this study, the only answer was the total flavonoid content (TFC). Thus, in Table 4 below are recorded the experimental design, the experimental responses and the calculated responses. It appears that there is no significant difference at the 5% threshold between the experimental responses and the theoretical responses. It is observed that the TFC are between (299.83-529.01) µg QE/g DW. The maximum recovery of TF was recorded under these experimental conditions of irradiation time X1=90 s at the irradiation power X2=700 W, at the liquid-to-solid ratio X3= 20/07 mL/g and at the methanol concentration X4= 30 %. Thus using the centered composite design (CCD), we evaluated more than a single effect of irradiation time, irradiation power, liquid-to-solid ratio, methanol concentration, the interactions and the quadratic effects of those factors on MAE on the recovery of total flavonoids. The experimental design, corresponding response data and predicted data for TF content, from the root bark of *T. emetica*, are given in Table 4. Second-order polynomial models for the flavonoids are developed by multiple regression analysis. The general form of the models is presented in Eq. (1), and the linear, quadratic and interactive coefficients for each model in terms of actual factors are given. The significance and adequacy of the model are assessed using analysis of variance (ANOVA) and the results show that this model is highly significant ($p < 0.01$), which suggests the model is adequate and reliable. The R-squared values of the model are above 95%, indicating that the predicted values correlate well with the experimental data. By applying multiple regression analysis on the experimental data, the mathematical model representing the recovery of TFC as a function of the independent variables within the region under investigation was expressed for the different responses. The regression coefficients of the linear, quadratic and interaction terms of the model were calculated using the least square technique (Zhang *et al*., 2013) and presented above in table 5. The single effect X1, X2, X3, X4, the interaction X1X3, X2X3 and the squared term of $X1^2$ were highly significant ($p<0.01$) and positive, which means that their increase increased the TFC, yield. The interactions X1X4, X2X4, X3X4 and the squared term of $X3^2$ were also significant but negative, which means that by decreasing them it is possible to decrease the extraction yield. The second-order polynomial equation calculated for the microwave-assisted extraction of total flavonoids is given above.

This result is in accordance with Table 6 which presents the different p-values of the direct effects, interaction and quadratic effects of factors and fig. 4A, B, C and D show the response surface analysis for the different interaction effects of the factors. A high temperature could cause, in addition to softening of the plant tissue, the breakdown of interactions between the desired metabolites and thus increase their solubility and therefore improve their diffusion speed, hence better yield (Gan and Latif (2011), from the model equations above, the validation parameters were determined and recorded in Table 6 and table 5 the validation parameters with ANOVA results.

Table 4: Experimental design with uncoded independent variables and the results.

No	Independent variables (actual values)				Experimental values	Predicted values
Run	X1	X2	X3	X4	Y_{TF} (µgQE/gDW)	Y_{TF} (µgQE/gDW)
1	70	500	0.5/20	30	299.83	294.56
2	90	500	0.5/20	30	340.04	353.94
3	70	700	0.5/20	30	337.17	343.15
4	90	700	0.5/20	30	387.14	371.66
5	70	500	0.7/20	30	292.36	295.26
6	90	500	0.7/20	30	426.20	437.50
7	70	700	0.7/20	30	408.96	423.56
8	90	700	0.7/20	30	529.01	534.92
9	70	500	0.5/20	50	465.83	449.09
10	90	500	0.5/20	50	435.39	429.63
11	70	700	0.5/20	50	423.90	421.43
12	90	700	0.5/20	50	384.84	371.10
13	70	500	0.7/20	50	290.06	314.38
14	90	500	0.7/20	50	394.60	377.78
15	70	700	0.7/20	50	391.16	366.42
16	90	700	0.7/20	50	384.84	398.95
17	63.928	600	0.6/20	40	408.39	408.33
18	96.071	600	0.6/20	40	479.04	482.19
19	80	439.2	0.6/20	40	366.46	360.65
20	80	760.7	0.6/20	40	407.82	416.72
21	80	600	0.4393	40	269.39	293.06
22	80	600	0.7609	40	336.59	316.01
23	80	600	0.6/20	23.92	403.79	381.80
24	80	600	0.6/20	56.07	371.63	396.72
25	80	600	0.6/20	40	370.48	373.69
26	80	600	0.6/20	40	373.93	373.69
27	80	600	0.6/20	40	377.37	373.69
28	80	600	0.6/20	40	377.37	373.69

X1: Irradiation time (s), X2: Microwave power (W), X3: Liquid-to-solid ratio (mL/g) and X4. Methanol concentration (%); YTF (µgQE/gDW): Recovery of total flavonoids

Second-order polynomial models for the flavonoids are developed by multiple regression analysis. The general form of the models is presented in Eq. (1), and the linear, quadratic and interactive coefficients for each model in terms of actual factors are given. The regression coefficients of the linear, quadratic and interaction terms of the model were calculated using the least square technique (Zhang *et al*., 2013) and presented below. The second-order polynomial equation calculated for the microwave-assisted extraction of total flavonoids is given below.

$$Y_{TF} = 373.696 + 22.977*X_1 + 17.4417*X_2 + 7.13821*X_3 + 4.64051*X_4 + 27.7085*X_1^2 + 20.714*X_1*X_3 - 19.7089*X_1*X4 + 19.9244*X_2*X_3 - 19.0626*X_2*X_4 - 26.7734*X_3^2 + 33.8533*X_3*X_4$$

YTF (mg QE/gDW): Recovery of total flavonoids

This result is in accordance with Table 6 which presents the different p-values of the direct effects, interaction and quadratic effects of factors and fig. 5A, B, C and D show the response surface analysis for the different interaction effects of the factors.

Table 5: **Regression coefficients and ANOVA results.**

Validation indicators	Y_{TF}	Standard value
R^2(%)	96.86	100
R^2adjusted (%)	97. 66	100
AMDA	0. 030	0
Bias factor (Bf)	1. 000	1
Accuracy factor (Af_1)	1. 031	1
Accuracy factor (Af_2)	1. 007	1

Bf= Af1= Af2= 1 and AADM= 0, R^2: Determination Coefficient, AMDA: Absolute Mean Deviation Analysis, YTF: Recovery of total flavonoids.

3.5 Response surface analysis

The response surface curves below show an effective interaction of quadratic and linear effects between the different independent variables. Moreover, it presents the different influences of the factors on the studied response, namely the total flavonoids content Fig. 5A to Fig. 5 D. It appears that the cumulative effect of liquid to solid ratio X_3 and irradiation power X_2 linearly causes a progressive release of total flavonoids up to a maximum, after which a decrease in the content was observed Fig.4A. In addition, the response surface curves below show an effective interaction of quadratic and linear effects between the different independent variables. Moreover, it presents the different influences of the interaction effects of the factors on the studied response, namely the total flavonoids content Fig. 4A to Fig. 4 D. It appears that the cumulative effect of liquid to solid ratio X3 and irradiation power X2 linearly causes a progressive release of total flavonoids up to a maximum, after which a decrease in the content was observed Fig.4A. These results is in accordance with the model equation shows that X2X3 are significant for the TFC microwave extraction with the p-value of 0.0017. The interaction effect of irradiation time X1 and liquid to solid ratio X3 linearly also causes a progressive release of total flavonoids Fig.4B. Hence X1X3 are significant for the TFC microwave extraction with the p-value of 0.0013. Methanol could facilitate an increase in the extraction yields and water could upgrade the swelling of vegetal cells, favourably increasing the surface contact area between plant material and solvent, increasing the extraction yield. Moreover, Fig. 4D in accordance with Table 5 shows that X3X4 are highly significant for the TFC microwave extraction with the very low value of the p-values which indicated that the fitness of the model was extremely significant. The p-values of the model for TFC recovery were 0.0001 for X3X4 which indicated that when correlating the methanol polarity X4 and liquid-to-solid ratio X3 together in Fig. 4D, the two factors interact to increase total flavonoid content. This result is in accordance with the model equation. When correlating irradiation power to irradiation time X1X2 Fig 4C, the extraction time decreases as we start to increase the irradiation power. Thus the large value of the irradiation power would lead to the degradation of the total flavonoids. The single effects of irradiation power tend to increase the extraction of total flavonoids. According to Gan and Latiff (2011), a high temperature could cause in addition to the softening of the plant tissue, the breakdown of interactions between the needing metabolites thus improving their solubility and therefore their diffusion speed and hence a better extraction yield. The methanol concentration influences both the extraction and other factors because the hydroalcoholic mixture is more suitable with better yield. Liu *et al*. found in their work (2011) that 70% methanol is ideal for the MAE of total flavonoids from *Radix rueraria thomsonii*. Moreover according to the optimization of extraction conditions of the polyphenols, and the flavonoids from the *Ammosperma cinereum* plant aerial part by the response surface methodology using the Box Behnken design, Bouaziz found that the duration and the methanol proportion positively affect the extraction of TFC (Bouaziz *et al.*, 2020). At the end of these analyses, it appears that nine effects significantly influence the extraction of TFC at the 5% threshold (Table 6). Among these nine factors, the five which contribute to increasing the extraction of total flavonoids are; the direct effects of irradiation time X1 and irradiation power X2, the interaction effects of irradiation time and liquid-to-solid ratio (X1X3), irradiation power and methanol concentration (X2*X4). However, the four independent variables that tend to reduce them are; the quadratic effects of irradiation power (X2*X2), of liquid-to-solid ratio (X3*X3) and methanol concentration (X4*X4), the interaction effects of liquid-to-solid ratio with methanol concentration (X3X4). The quadratic effect of time (X1*X1) linearly causes a progressive release of TFCs. In general, the greater amount of solvent has a positive effect on extraction yield due to more contact between the sample and the solvent, as it can dissolve and extract more plant components (Ismail *et al.*, 2019).

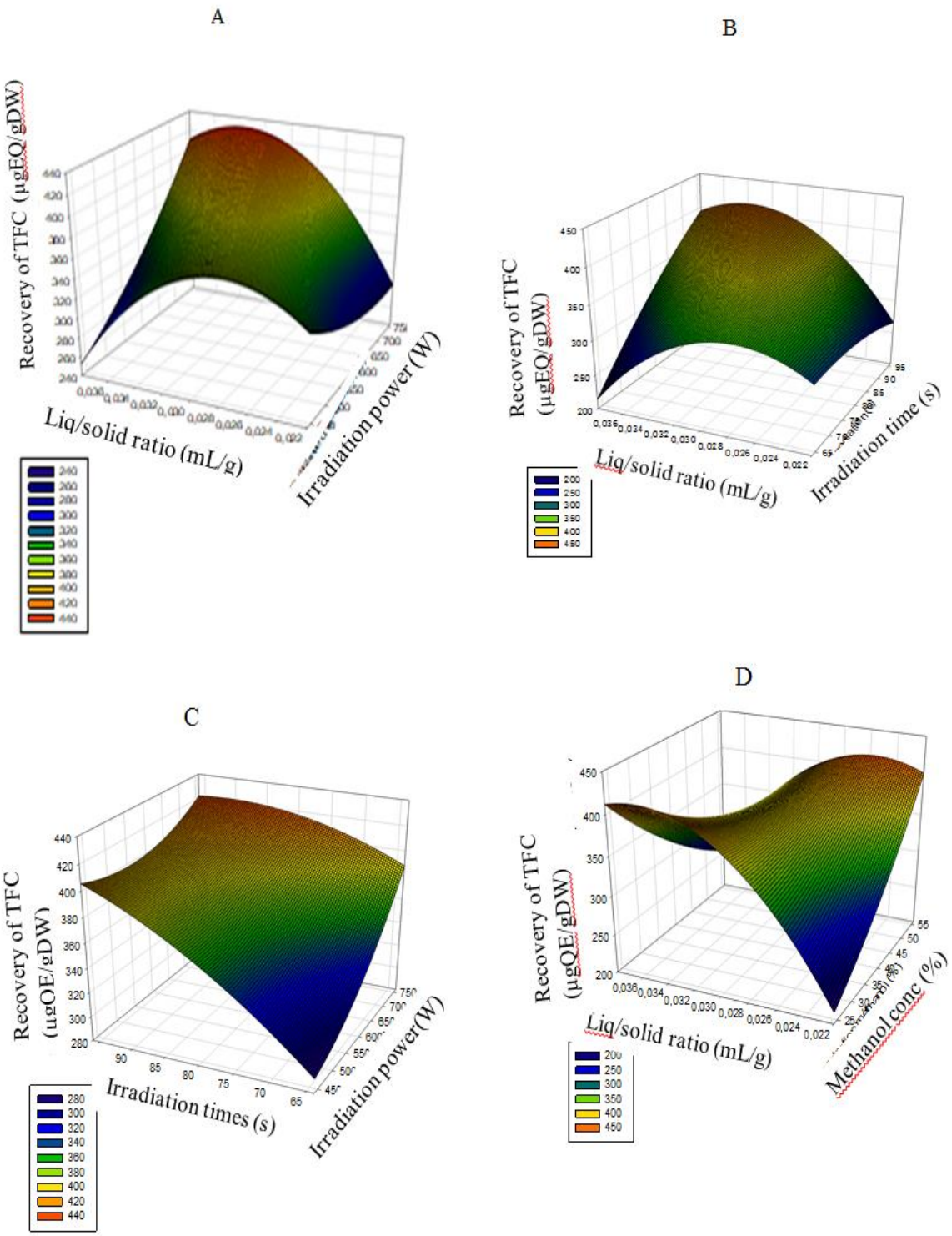


Fig. 4A. Liquid-to-solid ratio and irradiation power 3D plot; X_4 and X_1; fixed at 95% and 60 s respectively.
B. Liquid-to-solid ratio and irradiation time 3D plot; X_4 and X_2; fixed at 95% and 600W respectively.
C. Irradiation time and irradiation power3D plot; X_4 and X_3; fixed at 95% and 20:1 mL/g respectively.
D. Liquid-to-solid ratio and methanol concentration; X_1 and X_2 fixed at 60 s and 600 W respectively.

Table 6: Analysis of the variance (ANOVA) for the experimental results.

Factors		YTF
	Coeff	**P-value**
X1(Irradiation time)	11174.4	0.0002•
X2 (irradiation power)	6439.00	0.0016•
X3 (Liq/solid ratio)	1078.49	0.1289
X4 (methanol conc.)	455.80	0.3110
X1*X1	10244.80	0.0002 •
X1*X2	953.21	0.1513
X1*X3	6865.14	0.0013 •
X1*X4	6215.07	0.0019 •
X2*X2	449.61	0.3142
X2*X3	6351.69	0.0017 •
X2*X4	5814.15	0.0024 •
X3*X3	9564.99	0.0003 •
X3*X4	18336.80	0.0000 ••
X4*X4	484.70	0.2967

P<0.05, • significant, P< 0.0001, •• highly significant

4. CONCLUSION

This study has as objectives to analyze the extraction characteristics of flavonoids from *T. emetica* under microwave, to determine the optimum values of the parameters leading to the highest flavonoids extract yield, and to determine the best antioxidant percentage in the total flavonoid recovered. The results illustrate that the total flavonoid contents obtained by microwave-assisted extraction were twice those obtained by conventional solvent extraction. A second-order polynomial model could be employed to obtain the maximum total flavonoids. It was also demonstrated that, the optimal conditions amongst the four parameters tested were evaluated, and different antioxidant investigations by different methods took place. Extracts obtained by microwave-assisted extraction showed high efficiency, chelating power, DPPH radical scavenging and antioxidant activities using β-carotene oxidative degradation with inhibition values of 86, 90.36, and 82.49 % respectively. The predicted optimal conditions are as follows: microwave extraction time of 90 s, microwave extraction power of 700 W, liquid-to-solid ratio 20:0.7 (mL/g), and methanol concentration of 30%. The predicted and observed responses at the optimal conditions are listed in Table 4. Analysis of variance (ANOVA) was conducted to determine the statistical significance of factors; more experiments were performed to verify the predicted optimal conditions. In the experimental conditions, the extraction time was adjusted to 90 s and the other parameters were kept the same as the predicted values. The predicted values for the total flavonoid recovery are in close agreement with the experimental results, indicating that the predictive performance of the established models is reliable. MAE was identified as the best extraction approach for total flavonoids microwave extraction from *T. emetica* root bark and also showed great potential for industrial application to the extraction of other antioxidants secondary metabolites from plants. Hence a considerable advance in the fight against degenerative pathologies using *Trichilia emetica* root bark using and green extraction method with a positive environmental impact.

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