

PAPER 60 – EXPERIMENTAL PROCESS OPTIMIZATION AND GC-MS CHARACTERIZATION OF BIOACTIVE ANTIOXIDANT EXTRACT PRODUCTION FROM HYPPTIS SUAVEOLENS LEAVES

Aguele, Felix Osarumhense¹, Oke, Emmanuel Olusola^{1*}, Abam, Fidelis I.²

¹Department of Chemical Engineering, Michael Okpara University of Agriculture, Umudike (MOUUAU), Nigeria.

²Department of Mechanical Engineering, University of Calabar, Nigeria.

*Email: oke.olusola@mouau.edu.ng

ABSTRACT

Previous studies documented the biological and antioxidant activities of phenolic bioactive extract (phytochemical) from Nigerian *Hyptis suaveolens* leaves. However, optimization of solid-liquid extraction of the process for possible scale-up process design is seldom reported in the literature. Therefore, this investigation presents process optimization and characterization of bioactive extract recovery from *Hyptis suaveolens* leaves. Solid-liquid extraction experiment was designed at ranging extraction time (30 - 70 min), temperature (60 -75 oC) and substrate/solvent ratio (0.4 – 0.8 g/100ml) using Box-Benken Design (BBD) in Response Surface Methodology (RSM) of Design Expert software V13 with Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and extraction yields as dependent variables. BBD desirability algorithm was used to perform optimum conditions that optimized TPC, TFC and extraction yield. At the optimized process conditions of 61.28 mins, 62.74 oC and 0.58 g/100ml, the extract obtained gave TPC (33.055 mg GAE/g d.w.), TFC (7.151 mg QE/g d.w.) and yield (18.203%). The Gas Chromatography - Mass Spectroscopy (GC-MS) analysis of extracts recovered was observed to have a wide array of compounds, dominated by methyl esters (about 43%), ethyl-oleate (about 16%), acids (about 13%) and many other components at lower levels. The Fourier Transform Infra-Red (FT-IR) analysis indicated the presence of functional groups, which are attributable to antioxidants.

KEYWORDS: Optimization, Bioactive, Antioxidant, Extraction, *Hyptis suaveolens*, GC-MS

1. INTRODUCTION

Hyptis suaveolens (L. Poit) is an essential medicinal plant also known as bush tea, belonging to the family lamiaceae (Umedum et al., 2014). The plant has been reported to be traditionally utilized as an antispasmodic against diarrhea and colds (Singh et al., 2011) while the leaves are used to repel mosquitoes (Vongsombath et al., 2012) due to the presence of bioactive compounds in the plant. Extraction process is the crucial step to adequate utilization of the bioactive compounds (Kamarudin et al., 2020). However, the efficiency of the extraction process highly depends on the extraction techniques adopted (Aybastier et al., 2013). Due to the simplicity and efficiency of the heat assisted extraction (HAE) technique, it has been employed by researchers for the extraction of bioactive compounds from different plant materials (Ekezie et al., 2017; Oke et al., 2022). Jovanovic et al., (2023) reported the extraction and optimization of bioactive compounds from *Gentiana asclepiadea* L. Bankova et al., (2021) inferred that the HAE process gave high product yield with reduced process time and solvent consumption than the traditional extraction techniques. The efficiency of the extraction process is determined by the process parameters such as extraction time, temperature and substrate/solvent ratio (Alara et al., 2018a). The solubility and diffusion rate of the phenolic compounds in a plant is influenced by the extraction temperature (Santos et al., 2012) while the mass transfer of bioactive compound during extraction is product of the extraction time (Oke et al., 2020). Simultaneous evaluation of multiple responses in a process is an essential feature of the response surface methodology (RSM) as it mathematically depicts the interaction effects of the process parameters as well as the optimal values for the process (Ibrahim and Elkhidir, 2011; Azahar et al., 2017). Therefore, the RSM technique was employed to study the interaction effects of extraction time, temperature and substrate/solvent ratio on the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and extraction yield of *Hyptis suaveolens* leaves extract. Identification of the bioactive antioxidants of the

leaves extract was quantified through GC-MS and FT-IR analysis to determine the functional group present in the extract.

2. METHODOLOGY

2.1 Plant materials and Chemical Reagent

The fresh plant material (*Hyptis suaveolens*) was obtained from some botanical gardens in Ogbomosho, Oyo State, Nigeria. Other reagents and chemicals such as Gallic acid, Folin–Ciocalteu, ethanol and anhydrous sodium carbonate, quercetin, chlorogenic acid, caffeic acid, sodium hydroxide, aluminum chloride, potassium acetate, and rutin samples used in this study were analytical grade products of Sigma-Aldrich, Germany. They were obtained from various reliable chemical vendors and were used as obtained, without modification. Distilled water was obtained from Chemistry department laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria.

2.2 Preparation of Substrate and Extraction

The fresh materials (*Hyptis suaveolens* leaves) were plucked off the plant stems, washed, and dried in an aerated environment shaded from direct sun light, at a temperature of about 30°C for six (6) days. The dried leaves were ground to obtain fine particles and to enhance effective extraction. The ground material was then preserved properly in a compartment at 4°C before extraction was done. The extraction was done in a water bath, using appropriate solvent mixture and parameters according to the design. The parameters ranges were time (30 – 70 min), temperature (60 – 75 °C) and substrate/solvent (S/L) (0.4 – 0.8 g/100ml). The extracts were filtered using Whatman filter paper No 1, evaporated and properly preserved at 4°C for further analyses. The yield was calculated using Equation 1.

$$Yield = \left(\frac{\text{Mass of Bioactive Extracts obtained}}{\text{Mass of dried ground leaves used}} \right) \times 100\% \text{ ----- } 1$$

2.3 Determination of Total Phenolic Content (TPC)

The TPC of *Hyptis suaveolens* extract was determine, using Folin-Ciocalteu’s (FC) phenol reagent, adopting the procedure described by Alara and coworkers with slight modifications (Alara et al., 2018a). A small volume of distilled water was taken and topped up to 1 ml by adding 100 µl of extract. Next, 0.2 ml of the FC reagent was then added, thoroughly mixed and incubated in the dark at room temperature for 5 minutes. Then 0.6 ml of 0.2 mM of Na₂CO₃ solution was added and incubation was again done at room temperature in the dark for a period of 120 minutes. Then the absorbance was measured at 765 nm with a UV–VIS Spectrophotometer (Hitachi U-1800, Japan). Using different concentration solutions of gallic acid (50–500 mg/ml), a standard calibration curve was prepared. TPC was then expressed as mg gallic acid equivalents per gram of dried weight sample (mg GAE/g d.w.). The TPC of the extracts were then obtained using Equation. The analyses were done in triplicate and water was used as the blank.

$$TPC = \frac{\text{Concentration} \left(\frac{\text{mg}}{\text{ml}} \right) \times \text{solvent volume used (ml)}}{\text{Weight of dried sample (g)}} \text{ ----- } 2$$

2.4 Determination of Total Flavonoid Content (TFC)

To determine the TFC in the *Hyptis suaveolens* leaves extract, the methodology previously reported by other researchers was adopted with a little modification (Alara et al., 2018). 100 µL of 2% AlCl₃ solution was added to 100 µL of the *Hyptis suaveolens* extract, mixed vigorously and then kept at room temperature for 60 minutes. The absorbance was measured at 420 nm using a UV-vis Spectrophotometer (Hitachi U-1800, Japan). The quercetin acid standard calibration curve was prepared using different concentration solution of quercetin acid (50 - 500 mg/l). Then, the concentration of TFC in the extract was calculated with equation of line $y = 10.39x + 0.305$, $R^2 = 0.9941$ (where y is the absorbance at 510 nm and x is the concentration of TFC from the calibration curve), TFC was determined using Equation 3, and results were reported as quercetin equivalent in milligram per gram of dried weight sample (mg QE/g d.w.).

$$TFC = \frac{\text{Concentration} \left(\frac{\text{mg}}{\text{ml}} \right) \times \text{solvent volume used (ml)}}{\text{Weight of dried sample (g)}} \quad \text{-----} \quad 3$$

2.5 Experimental Design and Analysis

The extraction parameters were studied using three-factor, three-level box behken design (BBD) of response surface methodology (RSM) in design expert software V13, which gave 17 experimental runs. The parameters studied include time (minutes), temperature (°C) and substrate/solvent ratio (g/100ml). The experimental design is presented in Table 1. Second order polynomial equations were used to study the relationships between the process parameters and the responses of TPC, TFC and extract yield, as expressed in equations 3, 4 and 5, respectively.

2.6 GC-MS Analysis

The Hyptis suaveolens leaves extract was subjected to GC-MS investigation for constituent profiling. The Gas Chromatography equipment (Agilent 6890 series) equipped with HP-5MS column mass spectrometer was used. The procedure described previously by Kulkarni and coworkers (Kulkarni et al., 2015) was adapted. During the analytical operation, the initial column temperature of 30 °C was raised to 300 °C at a rate of 10 °C /5min. High purity helium flowing at 1.0ml/min was used as carrier gas. Compounds identification was done in accordance with the retention time and integral area of peaks. Based on the NIST library search, the similarity of compounds match with > 70% are listed.

2.7 FT-IR Analysis

FT-IR analysis was employed to characterize the extract obtained from Hyptis suaveolens. The procedures reported in previous researches were adapted (Alara et al., 2018b; Kumar and Thampi, 2015). The Hyptis suaveolens extract was subjected to spectrophotometer infrared scanning (Agilent Technologies, Cary 630 FTIR), employing wave number in the region between 4000-650cm⁻¹, which registered the characteristic peaks during the tests. The detected characteristic peak values and their functional groups of the FT-IR were recorded. The IR chart was used to interpret the infrared absorption frequencies of functional groups spectra obtained for the extract.

Table 1: Summary of Experimental Design Factors and levels

Factors	Level		
	-1	0	+1
A: Extraction Temperature (°C)	60	67.50	75
B: Extraction Time (minutes)	30	50	70
C: Substrate/Solvent ratio (S/L) (g/100ml)	0.4	0.6	0.8

3. RESULTS AND DISCUSSION

3.1 Experimental result and model fittings

The extraction experiment for the *Hyptis suaveolens* leaves comprised three process parameters: extraction temperature, time, and substrate/solvent ratio (S/L) resulting in 17 experimental runs using the BBD design. The experimental values of the TPC, TFC, and extraction yield at various extraction conditions are presented in Table 2. The TPC values varied from 19.45 to 43 gGAE/100g d.w., while the TFC varied from 4.75 to 11.17 gQE/100g d.w and the extract yield varied from 12 to 27%. The obtained optimal values of the bioactive compound were similar to the values obtained by Sanou *et al.*, (2023) from *Hibiscus sabdariffa* L. The experimental data subjected to multiple regression analysis depicted a second-order polynomial equation for each of the responses, TPC (equation 4), TFC (equation 5), and extract yield (equation 6).

$$TPC = +34.40 - 2.34A + 0.8238B - 7.81C + 2.00AB + 3.06AC - 2.02BC - 5.42A^2 - 4.25B^2 + 0.3850C^2 \quad (4)$$

$$\text{TFC} = +6.27 - 1.05A - 0.6350B - 0.6875C + 1.12AB - 0.5950AC - 2.03BC + 0.9260A^2 + 1.49B^2 + 0.5260C^2 \quad (5)$$

$$\text{YIELD} = +21.40 + 2.75A + 2.75B + 1.75C + 4.25AB + 0.7500AC - 1.25BC - 1.58A^2 - 2.58B^2 + 2.43C^2 \quad (6)$$

The statistical significance of the regression models was depicted by the ANOVA models in RSM and presented in Table 3. The quadratic models showed a significant p-value of <0.0001, 0.0012, and 0.0118 for the TPC, TFC, and yield respectively at a 95% (p<0.05) level of significance. The lack of fit of the models; TPC (0.0531 gGAE/100g d.w.), TFC (0.3313 gQE/100g d.w), and yield (0.1163) were not statistically significant confirming the fitness of the models and their relationship with the process parameters (Kamarudin *et al.*, 2020). The high coefficient of determination (R²) values of 0.9835 gGAE/100g d.w. (TPC), 0.9456 gQE/100g d.w. (TFC), and 0.8907 (yield) corroborated the efficiency of the model equations for accurate predictions of the bioactive antioxidants whereas the agreement between the actual and predicted values of the models were indicated by the adjusted R² values (Mushtaq *et al.*, 2015).

Table 2: Experiment result

Run	Temp	Time	S/L	TPC	TFC	YIELD
1	75	50	0.4	33.16	8.25	25
2	67.5	30	0.8	24.24	10.87	23
3	67.5	50	0.6	33.98	5.83	23
4	67.5	70	0.8	23.04	4.75	26
5	67.5	50	0.6	34.47	7.12	22
6	60	30	0.6	29.33	11.17	17
7	75	30	0.6	19.68	6.67	12
8	67.5	30	0.4	34	7.75	14
9	67.5	50	0.6	35.06	5.63	19
10	60	70	0.6	25.79	8.45	14
11	67.5	50	0.6	33.32	6.59	22
12	75	50	0.8	21.84	5.25	27
13	60	50	0.4	43	9	19
14	75	70	0.6	24.13	8.43	26
15	60	50	0.8	19.45	8.38	18
16	67.5	70	0.4	40.88	9.75	22
17	67.5	50	0.6	35.17	6.17	21

Table 3: ANOVA model fittings of the bioactive antioxidants

	TPC	TFC	YIELD
F-value	46.45	13.52	6.34
p-value	< 0.0001	0.0012	0.0118
Std. Dev.	1.4	0.6672	2.24
Mean	30.03	7.65	20.59
C.V. %	4.66	8.72	10.89
R²	0.9835	0.9456	0.8907
Adjusted R²	0.9624	0.8756	0.7502
Predicted R²	0.7778	0.4919	-0.3361
Adeq Precision	21.4512	12.815	8.4308
Lack of fit	0.0531	0.3313	0.1163

3.2 Influence of the process parameters on the bioactive antioxidants

The combined effects of the process parameters on the bioactive antioxidants of *Hyptis suaveolens* leaves were depicted with the 3-D modelling of the RSM (Figures 1a-i). Figure 1a indicated that increase in temperature decreases the TPC value while increase in extraction time increases the TPC. Increasing the temperature and S/L ratio decreased the TPC as shown in Figure 1b, while Figure 1c indicated that increase in S/L ratio decreased the TPC and increase in time increased the TPC. Increase in extraction temperature surges heat activation which enhances the diffusion rate of soluble phenolic compounds from substrates, however beyond the certain heat threshold, thermal lability may occur which reduces the TPC yield (Rosello-Soto *et al.*, 2019). This finding concurred to the report of Kamarudin *et al.*, (2020) on the extraction of phenolic compounds from *Eleutherine bulbosa*. In Figure 1d, increase in extraction time decreased the TFC but increase in temperature elevated the TFC. Prolonged time exposure of flavonoid to heat, thermally degrades the bioactive compound, reducing its yield, however, increasing temperature improves the solubility of flavonoids (Chen *et al.*, 2007). Increasing the temperature decrease the TFC value whereas increase in the S/L ratio increased the TFC (Figure 1e). Due to the solubility of bioactive compounds in polar solvents, increasing the S/L ratio simultaneously increased the TFC yield (Bahtar and Annisa, 2018). Figure 1f showed that increase in extraction time and S/L ratio increased the TFC yield. Increasing the extraction time and temperature increase the extract yield (Figure 1g). Similarly Figure 1h indicated that increase in temperature and S/L ratio increased the extract yield and Figure 1i depicted a simultaneous increase in yield as the extraction time and S/L ratio increase.

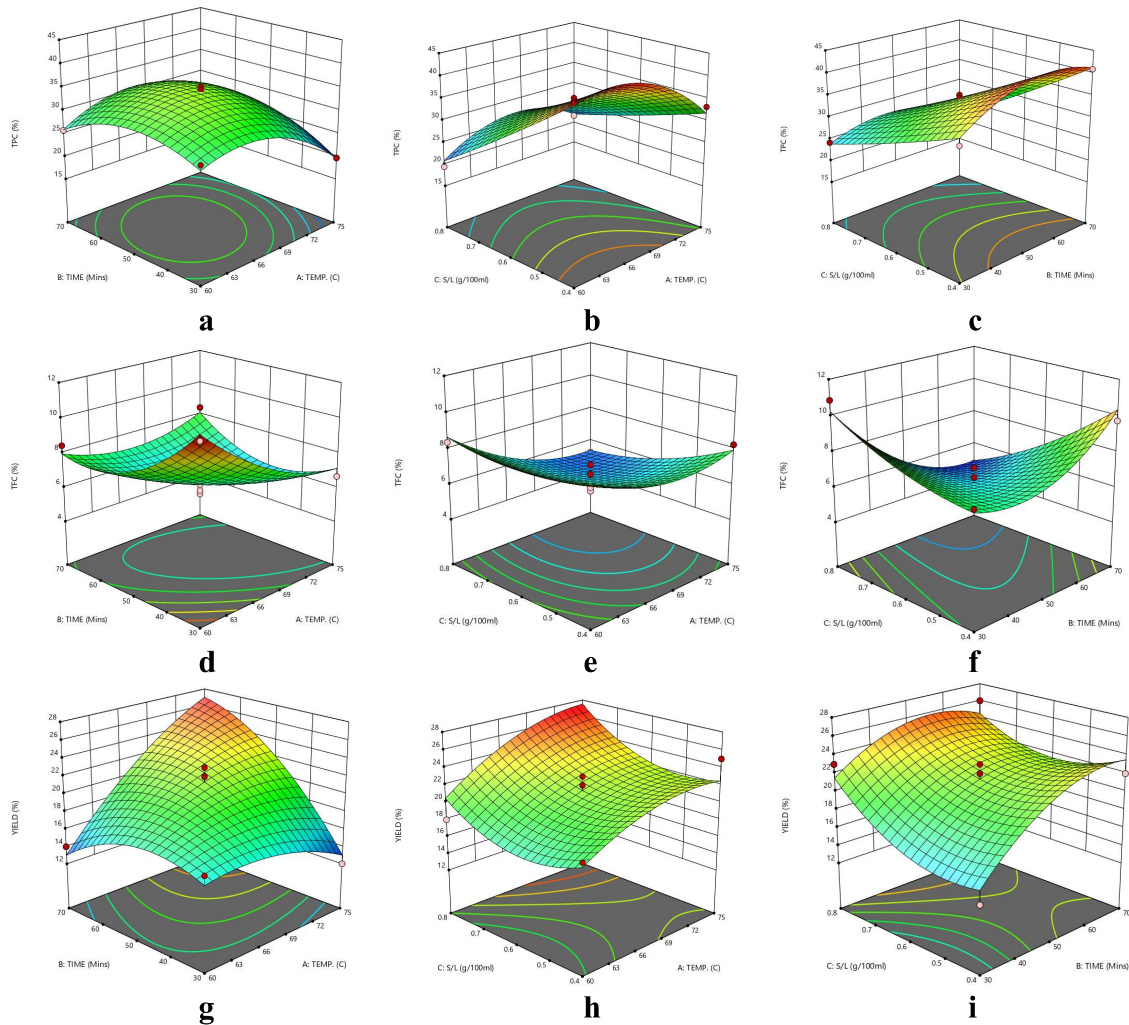


Figure 1: Effect of extraction time, temperature and substrate/solvent ratio on TPC (a-c), TFC (d-f) and yield (g-i)

3.3 Optimization studies of the bioactive antioxidants

Numerical optimization tool of Response Surface Methodology in Design Expert version 13 was used for optimizing the process parameters of the extraction process. The optimization was conducted “in the range” of the process parameters and responses. The optimization plot (Figure 2) depicted the optimal responses at the varied optimal process conditions. The optimal value of 33.055 mg GAE/100 g dw, 7.151 mg QE/100 g d.w and 18.203% were obtained for the TPC, TFC and yield respectively at optimal temperature of 62.74 °C, 61.28 minutes and S/L ratio of 0.58 g/100 ml at desirability value of 1. This inferred the agreement between the experimental and predicted values at confidence interval of 1%.

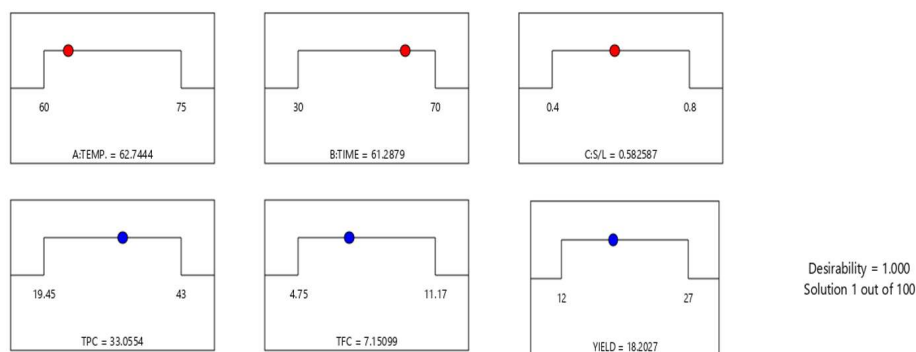


Figure 2: Optimization of the bioactive antioxidants extraction process

3.4 Characterization of *Hyptis suaveolens* leaves extract

The extract from *Hyptis suaveolens* leaves was characterized using Fourier Transform Infra-Red (FTIR) and Gas Chromatography – Mass Spectrograph (GC-MS). The GC-MS analysis of extracts recovered (Figure 3) was observed to have a wide array of compounds, dominated by methyl esters (about 43%), ethyl-oleate (about 16%), acids (about 13%) and many other components at lower levels. The identification of the functional group in the *Hyptis suaveolens* leaves using FT-IR (Figure 4) indicated strong peaks at 3339.7 cm^{-1} , 2974.4 cm^{-1} , 1043.7 cm^{-1} and 879.7 indicating the presence of O-H (hydroxyl group), CH_3 (lipid), C-O (lipid) and C-C (alkane) respectively. Other functional groups showed medium to weak peaks, indicating the presence of compounds, such as secondary alcohol, alkene, and amino compounds. These bioactive compounds were attributable to antioxidants and are responsible for the pharmacological activities of the plant extracts (Pakkirisamy *et al.*, 2017).

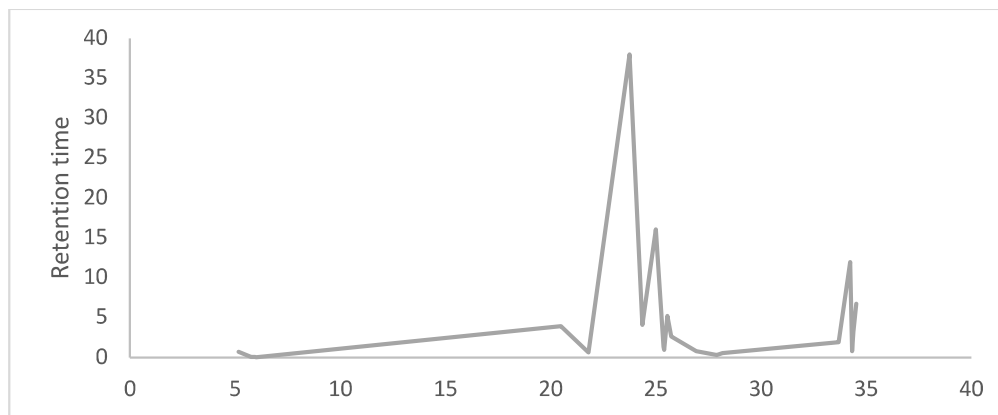


Figure 3: GC-MS spectrograph of *Hyptis suaveolens* leaves extract

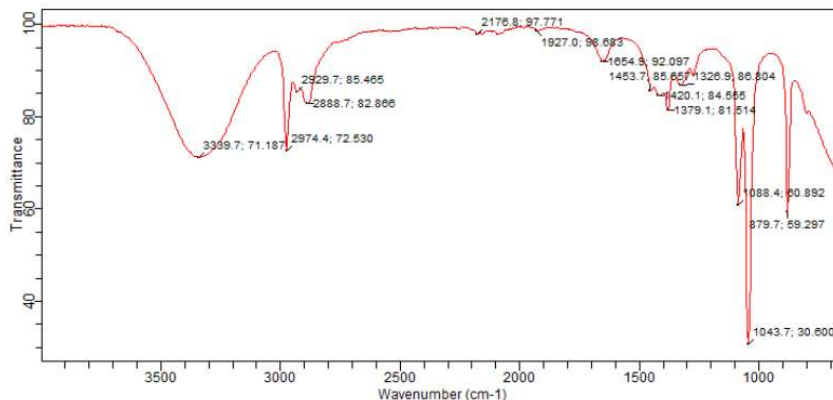


Figure 4: FT-IR analysis of the *Hyptis suaveolens* leaves extract

4. CONCLUSION

The conventional heat assisted extraction of bioactive antioxidants from the leaves of *Hyptis suaveolens* plants indicated optimal values of 33.055 mg GAE/g d.w., 7.151 mg QE/g d.w. and 18.203% for the TPC, TFC and yield respectively, at optimized process conditions of 61.28 minutes, 62.74 °C and 0.58g/100ml. The GC-MS analysis indicated that the *Hyptis suaveolens* leaves extract is dominated by methyl esters (about 43%), ethyl-oleate (about 16%), acids (about 13%) and many other components with lower values. The FT-IR analysis indicated the presence of functional groups, which are attributable to antioxidants.

REFERENCES

- Alara, O. R., Abdurahman, N. H., & Ukaegbu, C. I. (2018a). Soxhlet extraction of phenolic compounds from *Vernonia cinerea* leaves and its antioxidant activity. *Journal of Applied Research on Medicinal and Aromatic Plants*, 11, 12-17.
- Alara, O. R., Abdurahman, N. H., Mudalip, S. K. A., and Olalere, O. A. (2018b). Characterization and effect of extraction solvents on the yield and total phenolic content from *Vernonia amygdalina* leaves. *Journal of Food Measurement and Characterization*, 12(1), 311-316.
- Azahar, N.F., Gani, S.S.A., Mokhtar, N.F.M., (2017). Optimization of phenolics and flavonoids extraction conditions of *Curcuma Zedoaria* leaves using response surface methodology. *Chem. Central J.* 11 (1), 96.
- Bankova V, Trusheva B, Popova M (2021) Propolis extraction methods: a review. *J Apic Res* 60(5):734–743
- Ekezie FGC, Sun DW, Cheng JH (2017) Acceleration of microwaveassisted extraction processes of food components by integrating technologies and applying emerging solvents: a review of latest developments. *Trends Food Sci Technol* 67:160–172
- Ibrahim, H.M., Elkhidir, E.E., (2011) Response surface method as an efficient tool for medium optimisation. *Trends Appl. Sci. Res.* 6 (2), 121–129.
- Jovanovic, M. S., Milutinovic, M., Lazarevic, Z., Mudric, J., Matejic, J., Kitic, D. and Savikin, K. (2023). Heat- and microwave-assisted extraction of bioactive compounds from *Gentiana asclepiadea* L. underground parts: Optimization and comparative assessment using response surface methodology. *Journal of Applied Research on Medicinal and Aromatic Plants* (2023): 10048
- Jesus, N. Z. T., Falcão, H. S., Lima, G. R. M., Caldas Filho, M. R. D., Sales, I. R. P., Gomes, I. F., ... & Batista, L. M. (2013). *Hyptis suaveolens* (L.) Poit (Lamiaceae), a medicinal plant protects the stomach against several gastric ulcer models. *Journal of ethnopharmacology*, 150(3), 982-988.
- Kamarudin, A. A., Esa, N. M., Saad, N. and Sayuti, N. H. (2020). Heat assisted extraction of phenolic compounds from *Eleutherine bulbosa* (Mill.) bulb and its bioactive profiles using response surface methodology. *Industrial Crops & Products* 144 (2020) 112064

- Kulkarni, A., Govindappa, M., Ramachandra, Y.L., Koka, P., (2015). GC-MS analysis of methanol extract of *Cassia fistula* and its in vitro anticancer activity on human prostate cancer cell line. *Indo. Am. J. Pharm. Res.* 5, 937–944.
- Kumar, S. N. and Thampi, N. (2015,) Phytochemical screening and characterization of the bioactive compounds from the leaves of *Hyptis suaveolens* and *Spathodea campanulata* *J. Chem. Pharm. Res.*, 7(7):840-850
- Li, R., Tang, G., Liu, X., Li, J., Wang, D., & Ji, S. (2020). An ethnopharmacological review of *Hyptis suaveolens* (L.) Poit. *Tropical Journal of Pharmaceutical Research*, 19(7), 1541-1550.
- McNeil, M., Facey, P., & Porter, R. (2011). Essential oils from the *Hyptis* genus-a review (1909-2009). *Natural Product Communications*, 6(11), 1934578X1100601149.
- Mushtaq, M., Sultana, B., Bhatti, H. N. and Asghar, M. (2015). RSM based optimized enzyme-assisted extraction of antioxidant phenolics from underutilized watermelon (*Citrullus lanatus* Thunb.) rind. *J Food Sci Technol* (August 2015) 52(8):5048–5056
- Oke, E. O., Adeyi, O., Okolo, B. I., Ude, C. J., Okhale, S. E., Otolorin, J. A., Nnabodo, D., Ajala, O. O., Anyanwu, A. S. and Okorie, U. D. (2022). Polyphenolic Extract Recovery from *Azadirachta indica* Leaves via Green Microwave-Assisted Extraction Technology: GA-ANFIS Prediction, Cuckoo Search Algorithm Optimization and ASPEN-Based Scale-Up Techno-economics. *Process Integration and Optimization for Sustainability*
- Oke, E. O., Adeyi, O., Okolo, B. I., Adeyi, J. A., Ayanyemi, J., Osoh, K. A. and Adegoke, T. S. (2020). Phenolic compound extraction from Nigerian *Azadirachta Indica* leaves: Response surface and neuro-fuzzy modelling performance evaluation with Cuckoo Search multi-objective optimization
- Pakkirisamy M., Kalakandan S. K. and Ravichandran K. (2017) Phytochemical Screening, GC-MS, FT-IR Analysis of Methanolic Extract of *Curcuma caesia* Roxb (Black Turmeric). *Pharmacog J.* 9(6):952-6.7
- Roselló-Soto, E., Martí-Quijal, F. J., Cilla, A., Munekata, P. E., Lorenzo, J. M., Remize, F., Barba, F. J. (2019). Influence of temperature, solvent and pH on the selective extraction of phenolic compounds from Tigernuts by-products: triple-TOF-LC-MS-MS characterization. *Molecules* 24(4), 797.
- Raja, N., Jeyasankar, A., Venkatesan, S. J., & Ignacimuthu, S. (2005). Efficacy of *Hyptis suaveolens* against lepidopteran pests. *Current Science*, 88(2), 220-222.
- Sanou, A., Konate, K., Kabakde, K., Dakuyo, R., Bazie, D., Hemayoro, S. and Dicko, M. H. (2023). Modelling and optimisation of ultrasound-assisted extraction of roselle phenolic compounds using the surface response method. *Scientific Reports* 13: 358
- Santos, D.T., Veggi, P.C. and Meireles, M.A.A. (2012) Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from jabuticaba skins, *J. Food Eng.* 108 (3): 444–452
- Singh, V., Shrivastava, G. and Shukla, S. (2011). Mosquito repellent activity of essential oils of *Hyptis suaveolens*. *Journal of Pharmacy Research.* 4(8):2778–2779.
- Umedum, N. L., Ugochukwu, N. and Ifeoma, P. U. (2014). The efficacy of *Hyptis suaveolens*: A review of its nutritional and medicinal applications. *European Journal of Medicinal plants.* 4(6):661–674.
- Xu, D. H., Huang, Y. S., Jiang, D. Q., & Yuan, K. (2013). The essential oils chemical compositions and antimicrobial, antioxidant activities and toxicity of three *Hyptis* species. *Pharmaceutical Biology*, 51(9), 1125-1130.
- Vongsombath, C., Palsson K. and Bjork L. A, (2012). Mosquito (Diptera: Culicidae) Repellency Field Tests of Essential Oils from Plants Traditionally used in Laos. *Journal of Medical Entomology.* 49(6):1398–1404.