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Research Article

Exploring Microwave Hydrodiffusion and Gravity as an Eco-extraction Protocol for Phenolic Extraction from Lettuce: A Step Towards Green Sustainable Processes

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Abstract: The current work portraits an innovative approach in the field of botanical extraction using a zero solvent concept of phenolic extraction by implementing microwave hydrodiffusion and the gravity method. *Lactuca sativa* leaves were used for phenolic extraction without using any solvent and firing microwave power at different power levels (170-510 W). Total phenolic content was chosen as the performance evaluation parameter. Results indicated that a 340 W power level using the Microwave Hydrodiffusion and Gravity (MHG) protocol, comprising of 20 min of microwaving, produced a highly enriched phenolic extract (3,436.55 µg GAE/g of dried extract) when compared to a 24 h Soxhlet extract (496.36 µg GAE/g of dried extract). Phenolic profiling also revealed the richness of the extract produced from the MHG protocol. Improved biological potency by more than 48% in terms of antioxidant activity was observed in the extract produced from MHG. High-Performance Thin-Layer Chromatography (HPTLC) chromatograms revealed no formation of any undesirable adduct as well. The work is an innovative attempt in the field of green sustainable processes, which is the need of the hour for industries.

***Keywords*:** *Lactuca sativa*, microwave hydrodiffusion and gravity, phenolics, lettuce

1. Introduction

Extraction forms the starting element in the actual production line in a herbal drug industry (Zhang et al., 2018). Ideally, an extraction process should be fast, exhaustive, ecofriendly, safe for the phytoconstituents, automatized with precision involved, and should involve minimum operational steps (Chemat et al., 2012). Maceration and decoction are the most preferred models in herbal manufacturing units as they are the most trusted traditional process and are in practice since ages. Even though most of the above-mentioned ideal qualities of an extraction method are missing in these traditional methods but the greatest advantage of these methods is that they are mentioned in ancient books of traditional medicine and have been in practice for centuries (Hidayat & Wulandari, 2021). With artificial intelligence invading our lives and digitization having now become a new normal, one has to respond to the massive call of technological intervention in the field of herbal drug extraction.

Microwave Hydrodiffusion and Gravity (MHG) is a new upcoming promising technique that makes use of the

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principles of microwave heating and does not make use of any solvent in the process (Mukherjee et al., 2023). The inherent water present naturally inside the plant material absorbs microwaves and generates internal heat inside the plant cell. This internal heat stress compromises the cellulose content, which is the building block of the cell wall, thus jeopardizing its integrity and aiding in the leaching out of the target analytes. The MHG technique is a zero solvent method for the quick extraction of phytoconstituents and can be a boon to the herbal drug manufacturing units as it will rejuvenate and revitalize their lifeline which is "extraction".

Dietary polyphenols are nowadays becoming an essential commodity for a healthy living and has taken the shape of a million-dollar industry. The positive role of dietary polyphenols in maintaining the body's oxidative balance and slowing down the progression of various diseases-oriented complications is now well known and evident through various scientific findings (Xu et al., 2017). In this regard, lettuce (*Lactuca sativa*) is an important element in the arena of dietary polyphenols. Lettuce is globally used as a leafy vegetable, often consumed raw in salad form. Lettuce is rich in polyphenols such as Chlorogenic acid, Caffeic acid, Ferulic acid, Quercetin, Kaempferol, and Apigenin, and has also been reported to be beneficial in the management of cardiovascular disorders, diabetes, cancer, and neurodegenerative conditions due to its anti-oxidant, anti-inflammatory, and anti-carcinogenic properties (Kim et al., 2016).

Given the above situation, a strategic plan was adopted to develop an eco-extraction protocol driven by the MHG principle for the extraction of polyphenols from lettuce. In this regard, the MHG protocol was used to achieve the set goals in an eco-friendly manner without the use of any solvent.

2. Material & methods

2.1 *Biomass*

Lettuce (*Lactuca sativa*) was purchased from a local farmer in the month of November just at the start of the winter season. The plant material was thoroughly washed, excess water was drained and then used for the MHG protocol in fresh condition and for Soxhlet extraction in dried condition. It may be noted that it is a prerequisite in case of MHG to use fresh biomass, whereas, for traditional methods, dried biomass is used. Henceforth, based on the compatibility with the extraction method, fresh biomass was used for MHG, and dried biomass was used for Soxhlet extraction. The moisture content of fresh plant sample was determined using loss on drying method which was found to be 81.5 ± 5% before subjected to the extraction protocols. An identification number, *Bot/GGV/2024/143*, was allotted to the sample.

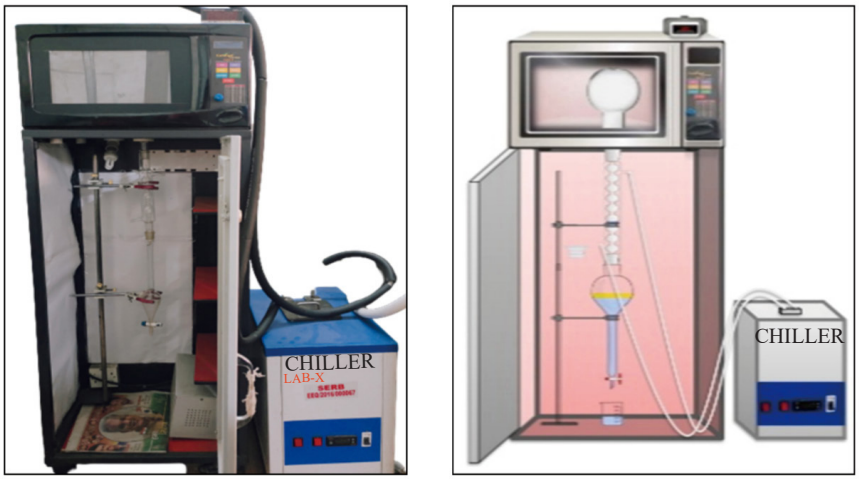
2.2 *Chemicals*

2,2-Diphenylpicrylhydrazyl (DPPH) was purchased from Sigma (St. Louis, MO, USA). Reagents like Folin reagent and solvent like methanol (Analytical Reagent (AR) grade), were procured from Central Drug House (CDH) Fine Chemicals (Mumbai, India).

2.3 *Apparatus*

MHG extraction was conducted using a Catalyst (CATA) R-invert microwave extractor, manufactured by Catalyst System, Pune (India). The system operates with a 2,450 MHz magnetron, delivering a maximum power of 850 W with adjustable power functions. The extractor also has a programmable timer, a powerful exhaust, and a beam reflector. The extraction vessel comprises of a 2 L sized oval glass vessel, which is loaded with fresh plant material. The vessel is kept in an inverted mode inside the microwave cavity, and a notch near the neck of the vessel prevents the biomass from falling down. The extraction vessel, in turn, connects with a glass condenser via an adapter, and the entire connection is toward the downward direction. The condenser further connects to a receiving flask (Florentine flask), where the final collection of the extract takes place. The entire process works in favor of gravity (Figure 1).

Conventional Soxhlet extraction was carried out for 24 h in methanol. This method was considered as a control and was compared with the performance of the proposed MHG method. After extraction, the sample was dried, re-dissolved in methanol, and used for quantitative phytochemical analysis. Chromatograms were studied using a CAMAG (Switzerland) High-Performance Thin-Layer Chromatography (HPTLC) system.



(a) (b)

Figure 1. MHG extraction model (a) Laboratory scale and (b) Schematic diagram

2.4 *MHG protocol*

Extraction was performed using 50 g of fresh chopped biomass. It may be noted that no organic solvent was used during the extraction. The extraction vessel was kept in an inverted position as shown in Figure 1. The mouth of the extraction vessel was protected by flower flower-shaped notch which prevented the falling of biomass when the vessel is kept in an inverted position. Microwave was fired at 170 W, 255 W, 340 W, 425 W & 510 W, in small cycles of 5 min each, with a cooling time of 2 min between successive irradiation. A fresh lettuce sample was loaded into the extraction vessel for each microwave power level. During microwave firing, the aqueous extract moves in the downward direction and collects in the receiving flask. Temperature of the biomass was measured after every microwave firing of 5 min using a handheld Infrared (IR) thermometer. Extraction endpoint was deemed to have reached when no visible collection of extract was observed. At the end of the extraction protocol for every power level, a sharp jet of water was sprayed on the biomass for 3 seconds to wash the analytes adhering to the vegetative surface of the biomass. The aqueous extract so collected was evaporated to dryness and then reconstituted in methanol.

2.5 *Soxhlet extraction*

Fifty grams of fresh plant material was dried under shade for 15 days, coarsely powdered, and then subjected to Soxhlet extraction with water circulated at 10 °C in the condenser through a laboratory recirculating chiller. The extract obtained was dried and reconstituted in methanol as stated above. To compare the phenolic content of the extracts obtained, moringa leaves were taken and extracted with the same protocol as mentioned above, and its extract was used as a reference standard due to its well-documented high phenolic content (Rani et al., 2018). The sole purpose of using moringa leaves was to understand how good the phenolic contents are for lettuce. Total phenolic content has no standard values through which the phenolic richness of the plant can be judged. Henceforth, moringa, which is also considered as a "superfood," was used as a reference standard for the purpose of comparison of phenolic richness between lettuce and moringa.

2.6 *Extraction yield*

The extraction yield of extracts obtained from Soxhlet extraction and MHG was calculated using the formula mentioned below:

$$\text{Extraction yield (\%) w/w in terms of fresh weight of biomass} = \frac{\text{weight of dried residue (g)}}{\text{weight of fresh biomass (g)}} \times 100 \quad (1)$$

2.7 *Total Phenolics Content (TPC)*

The procedure and calculation for determining TPC has been thoroughly described in the previous publications by the authors (Singh Chouhan et al., 2020). The TPC was performed using the Folin-Ciocalteu method with gallic acid as the standard. Briefly, 1 mg/mL of the sample were diluted in 5 ml of 10% Folin reagent and 4 ml of sodium carbonate solution (75 g/L). The above solution was vortexed and incubated for 1 h in the dark, and absorbance was recorded at 765 nm against methanol as blank using UV-Spectrophotometer (UV-1800). The result was expressed as Gallic Acid Equivalent (GAE) in µg/g of dried extract. The calibration curve, $y = 0.0094x + 0.1692$, $R^2 = 0.9992$, was prepared by using standard concentrations of gallic acid (5, 10, 20, 40, and 80 µg/mL) in methanol. TPC of the extracts was calculated using the following formula:

$$\text{Total Phenolic Content (µg GAE per gm of dried extract)} = \frac{C \times V \times df}{m} \quad (2)$$

Where,
c = value of x in the calibration equation obtained after putting the absorbance value;
v = volume of reaction mixture (10 mL);
df = dilution factor, if any;
m = weight of the dried extract (g).

2.8 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) based phenolic profiling

The extract of MHG and Soxhlet were analyzed for phenolic acids using LC-MS-MS as described by Weider et al. (2000) and Chen et al. (2001), with slight modification. Initially, the dried methanolic extract was dissolved in water and extracted three times using petroleum ether. The remaining aqueous phase was then extracted with ethyl acetate using a separating funnel. The water layer was discarded, and the ethyl acetate layer was concentrated by evaporation to dryness.

To the resulting dry material, 4 mL of 2(N) Sodium Gydroxide (NaOH) was added and allowed to hydrolyze overnight. Afterward, the mixture was acidified to pH 2 using 5 mL of 2(N) Hydrochloric Acid (HCl), and phenolics were again extracted using 10 mL of ethyl acetate. This enriched ethyl acetate fraction was dried under vacuum, and the final residue was re-dissolved in 1 mL of MS-grade methanol and filtered with the help of a 0.2 µm nylon syringe filter before injecting into the LC-MS/MS system for phenolic acids estimation (Mukherjee et al., 2024).

Analytical separation was performed utilizing a Waters BEH-C18 column (dimensions: 2.1 × 50 mm, particle size: 1.7 µm) coupled with a corresponding BEH-C18 guard column (Waters Corporation, USA). The chromatographic system operated under gradient conditions using two mobile phases: eluent A consisting of water containing 0.1% formic acid, and eluent B comprising methanol with 0.2% formic acid. The mobile phase delivery rate was set at 0.1 mL/min, while the column compartment temperature was controlled at 25 °C. The sample introduction was accomplished through 6 µL injections. Compound detection was achieved using photodiode array detection technology, with the chromatographic effluent being transferred directly to a Triple Quadrupole Tandem Mass Spectrometry system (TQD-MS/MS, Waters) without stream division. This analytical configuration was specifically tailored for the identification and quantification of phenolic compounds following established protocols for such analyses (Chen et al., 2001; Mukherjee et al., 2024; Weidner et al., 2000).

2.9 *HPTLC analysis*

HPTLC was utilized to generate phenolic fingerprint profiles of extracts obtained through both MHG (255 W, 340 W, & 510 W) and the Soxhlet extraction method. The analytical procedure followed was adapted from the methodology

previously reported by the authors' research group (Kala et al., 2017). A methanolic solution of lettuce extract was prepared (10 mg/mL) and applied to pre-coated silica gel 60 F_{254} aluminium plates. Chromatographic separation was conducted using a mobile phase composed of toluene: ethyl acetate: formic acid: methanol in the ratio of 6:6:1.6:0.4 (v/v). Densitometric scanning was performed at 366 nm in absorption mode, using a slit dimension of 6 image.jpeg 0.45 mm. The chromatographic data obtained were processed and evaluated using WinCATS software (version 1.4.4).

2.10 *Dot-blot analysis (TLC bioautography)*

It is the TLC bioautography technique employed for the detection of antioxidant compounds directly on the HPTLC plates themselves. For this purpose, silica gel precoated HPTLC plates (10 × 5 cm) were used. Lettuce extract (10 mg/mL in methanol), obtained through Soxhlet extraction and the MHG method, was applied onto the precoated HPTLC plates. Chromatographic development was carried out using a mobile phase as mentioned in the above section. After development, the plates were immersed in a 0.4 mM solution of DPPH in methanol, which stains the plate with a uniform purple colour. Spots containing antioxidant compounds were identified as yellow areas on the plate, resulting from the bleaching of the purple DPPH colour due to radical scavenging activity. These yellow spots were designated to symbolize antioxidant compounds as described by Singh Chouhan et al. (2019).

2.11 *Scanning Electron Microscopy (SEM)*

The samples which were used for micrography are as follows: (i) control: untreated dried lettuce leaves, (ii) leftover biomass post MHG extraction at the optimum microwave power level, and (iii) leftover biomass post Soxhlet extraction. Dried leaf samples were carefully mounted on copper stubs using carbon conductive tape to ensure proper conductivity. The sample surface was cleaned to avoid contamination and then coated with the palladium-platinum alloy using a JEOL 3000FC fine coater to enhance conductivity. These coated stubs were mounted onto stub holders and placed into the specimen chamber of a JEOL FESEM 7610F model. SEM images were captured at an acceleration voltage of 5 KV and recorded at various magnifications to reveal the surface morphological features of each specimen (Singh Chouhan et al., 2019).

2.12 *Statistical analysis*

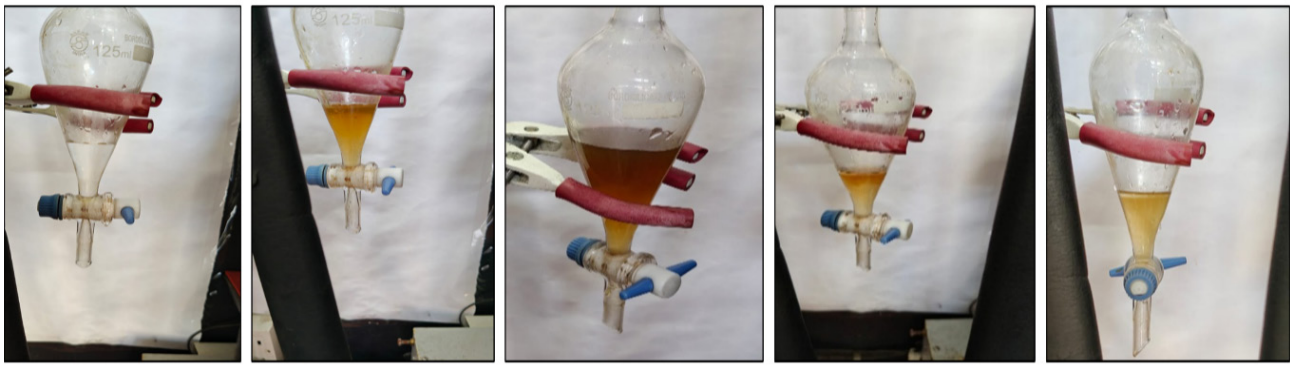
Student's t-test and the Duncan multiple range test were used to compare the means. $P < 0.05$ was considered significant. Experiments were conducted in triplicate, and the results were displayed as means and standard deviations.

3. Results and discussion

3.1 *Effect of microwave power and extraction time on MHG*

To obtain optimal performance, it is important to maintain the balance between low and high microwave power level. The authors have emphasised this balance in several of their previously published works (Chouhan et al., 2022, 2023; Mukherjee et al., 2023). Figure 2 represents the real time images of the aqueous extract collected at different microwave power level during the MHG extraction protocol. Visible observation clearly indicates that at power level 340 W, the extract appears darker in colour with increased volume. However, visible observation cannot yield conclusive evidence regarding the quality of the extract. Henceforth, the aqueous extract was dried, and a concentration of 1 mg/mL in methanol was prepared for TPC estimation.

Figure 3 indicates the extraction yield obtained from all the microwave power levels along with Soxhlet extraction used as a control experiment. Results clearly indicate that 340 W microwave power level produced highest yield which was found to be 36.5% more than Soxhlet extraction used as control. Once again, to mentioning that the yield of the extract also cannot provide conclusive evidence regarding the quality of the extract, which is only possible through quantitative phytochemical analysis. In light of the above facts, TPC was determined as the process evaluation parameter. The decision on optimal microwave power level was therefore taken based on the findings of TPC estimation.



(a) (b) (c) (d) (e)

Figure 2. Color of extract collected at different microwave power (a) 170 W, (b) 255 W, (c) 340 W, (d) 425 W and (e) 510 W

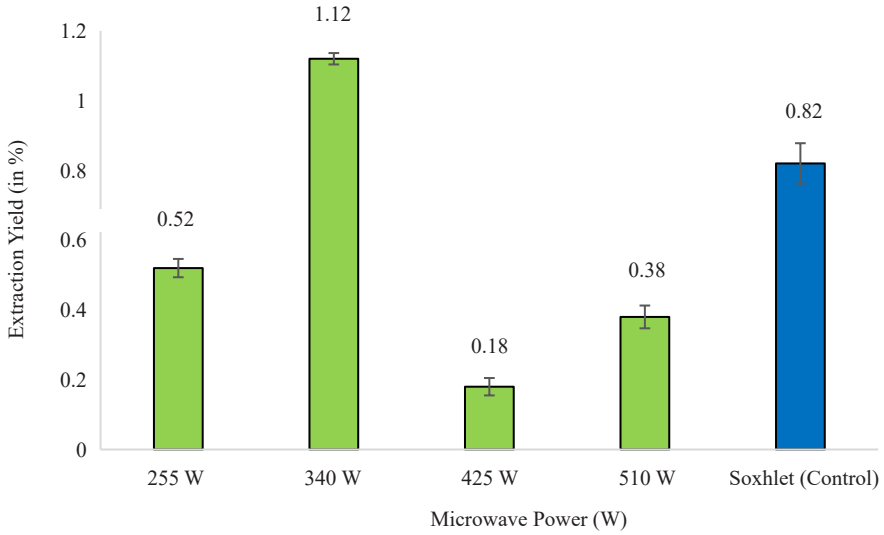


Figure 3. Impact of different microwave power levels on the extraction yield of L. sativa.
Data marked with different letters are significantly different at $p < 0.05$. Results are expressed as mean ± SD (n = 3)

Figure 4 indicates the TPC of the extract obtained from different MHG extraction conditions and Soxhlet extraction. Findings of TPC clearly demonstrate that the microwave power level of 340 W produces the highest phenolic content; the results are in sync with the findings of visible observation (Figure 2) and extraction yield (Figure 3), which also revealed 340 W as the optimal power level. The TPC extracted at a 340 W microwave power level was 6.9 times more than Soxhlet extraction. Given the absence of established standard values for total phenolic content to assess plant phenolic richness, the TPC of moringa extract was determined, and its value is used as a reference standard because it is well documented for its high phenolic content and is known as a "super food", and is compared with the phenolic content of lettuce. The result obtained represents that the TPC of lettuce extract obtained at 340 W microwave power level was found to be 5.34 times more than that of the TPC of moringa extract. This comparison with moringa extract clearly indicates that if the right extraction system is applied, then lesser explored polyphenolic-enriched plants can also be developed as drug/nutraceutical candidates for the herbal industry. The extraction pattern reflects a sharp spike type increase in phenolic content when the microwave power level was increased from 255 W to 340 W. Whereas, after 340 W, the opposite happens, where a sharp spike type decrease in phenolic content is seen. Microwaves are absorbed by the in-situ moisture content of the biomass, resulting in the generation of internal thermal stress causing degradation of the cellulose content of the cell wall, which is responsible for the strength and integrity of the cell wall. Moreover, due

to the internal heat generation, the in-situ moisture evaporates and exerts pressure on the cell wall. Both phenomenon contributes to cell wall degradation, leading to the development of fractures or pores, which in turn serves as the direct exist route for the analytes. Too less of microwave is incapable of generating enough thermal stress, thus unable to produce a significant impact on the plant ultrastructure and hence, extraction in totality cannot be achieved (Mukherjee et al., 2023). On the other hand, beyond 340 W, delivery of high electromagnetic energy creates tremendous heat stress capable of causing thermal degradation of the phytoanalytes, leading to their downfall in yield (Chouhan et al., 2022). Henceforth, in case of microwave-based extraction, striking a balance between high and low microwave energy for the optimal yield is the key to success.

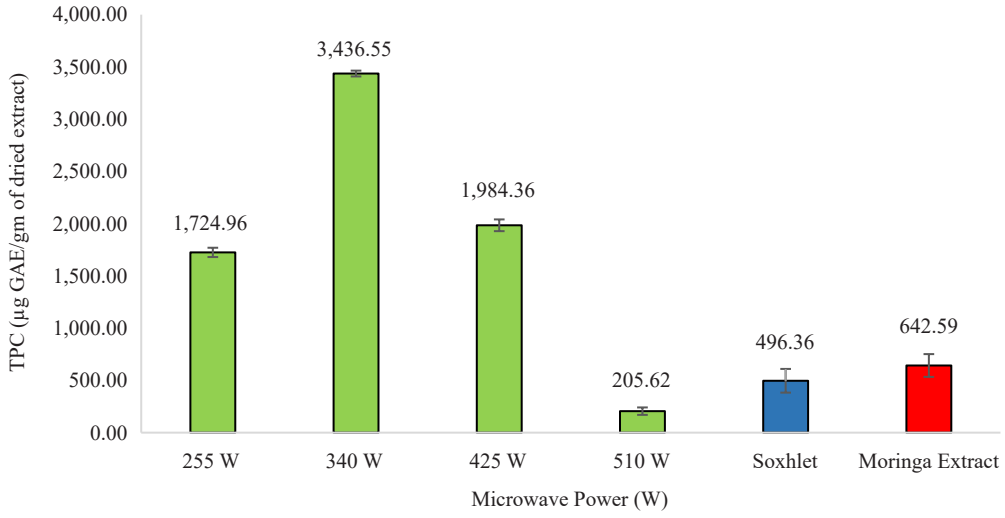


Figure 4. Extraction performance in terms of effect of various microwave power levels on the total phenolic content. Data marked with different letters are significantly different at $p < 0.05$. Results are expressed as mean ± SD (n = 3)

In a conventional extraction process, like Soxhlet and maceration extraction, the solvent slowly penetrates the rigid cell wall and solubilizes the phytoanalytes, which then diffuses out through the rigid cell wall due to the difference in concentration gradient. In the case of MHG, due to the impact of the microwave, the rigidity of the cell wall is compromised, and a direct exit route is made available for the phytoanalytes from the disrupted and compromised cellular structures. The vapour generated from the heating of the in-situ moisture content aids in the exit of the target analytes through diffusion. Similar observation of improved MHG performance over Soxhlet extraction was also reported by the authors in the past (Chouhan et al., 2022; Sinha et al., 2025). Findings also indicate that a severe depletion in phenolic contents to the extent of 16.7 times from the optimal microwave power level was observed when the microwave power level was increased upto 510 W (Figure 4).

Extraction time cannot be discussed in isolation and has to be understood in light of the findings of microwave power. Findings clearly indicate that with increasing microwave power, the extraction time follows a decreasing pattern. Extraction time is divided into short cycles of 5 minutes, and as microwave power increases, the requirement of the extraction cycle decreases. As more amount of microwave power is delivered to the system, internal heat stress and pressure build up quickly performs the evacuation action and completes the extraction (Chouhan et al., 2022). Microwave power being inversely proportional to the extraction cycle is a standard operational observation when dealing with microwave-based extraction and has also been reported earlier by the authors (Chouhan et al., 2022). MHG protocol with extraction duration in cycles and the sight of first drop for all microwave power levels is illustrated in Figure 5. Biomass temperature was also recorded during the extraction cycles for all microwave power levels which is presented in Figure 6. The extraction time when read together with the biomass temperature profile, it becomes evident that extraction reaches its optimal force when a best operating temperature is gradually reached which in this case is 84.6 °C. Microwave power levels below 340 W failed to reach this operating temperature throughout the extraction

cycle and hence could not reach the peak extraction force. Higher microwave power levels resulted in attainment of higher temperatures than 84.6 °C, but such attainment was very instant in the first cycle of extraction itself, which immediately exposed the biomass beyond the temperature comfort zone, which led to degradation. Gradual attainment of best operating temperature is the key to success. This specific temperature has been named by the authors as "critical temperature" in one of their previous publications. (Chouhan et al., 2022; 2023; Mukherjee et al., 2024)

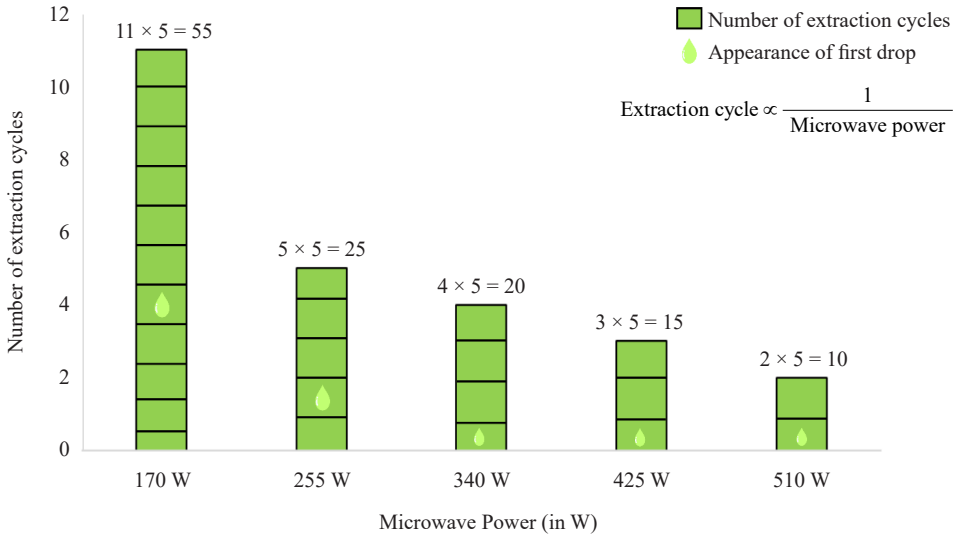


Figure 5. Number of extraction cycles along with first drop appearance for various microwave power levels

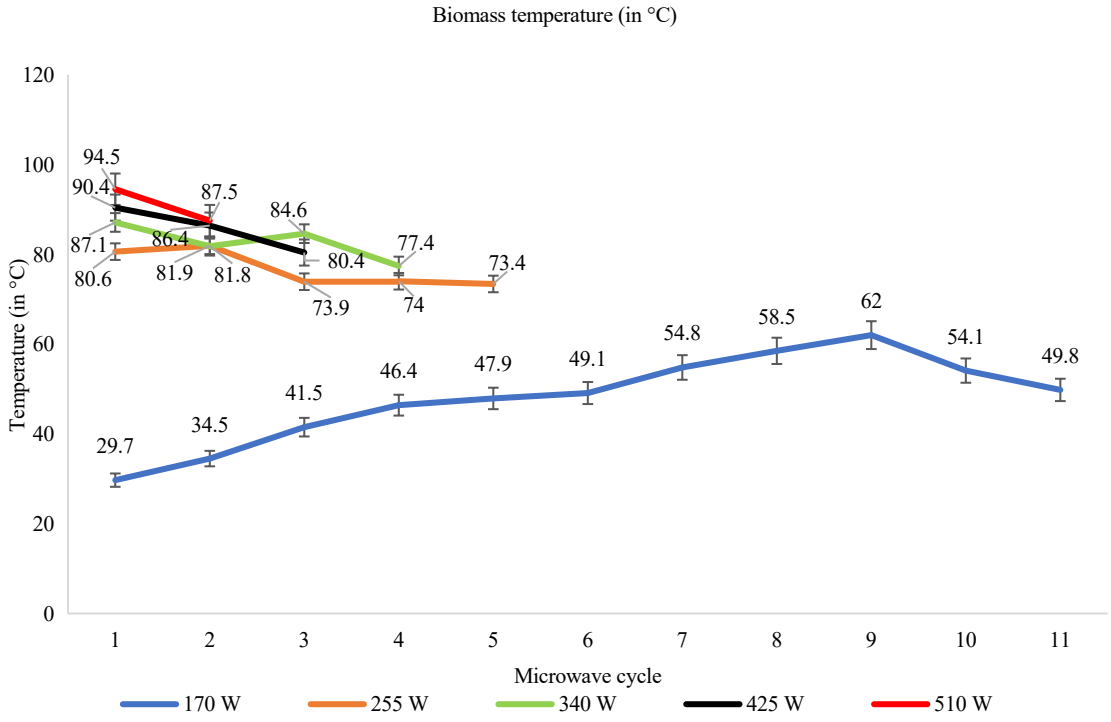


Figure 6. Effect of different microwave power levels on the temperature of biomass obtained at the end of each extraction cycle during MHG. Results are expressed as mean ± SD (n = 5)

3.2 *Phenolic profiling (LC-MS/MS)*

LC-MS/MS was used for complete phenolic profiling, which is the identification of the individual phenolic present in the extract obtained from MHG & traditional Soxhlet. The LC-MS/MS provided conclusive evidence regarding the quality of extracts through the identification & quantification of phenolics present. It was clearly evident from the findings that extract quality obtained from MHG was significantly better than obtained from Soxhlet in terms of quantity of phenolic compounds present. Noteworthy, to mention that HPTLC was used as a second-line confirmation of the quality of the extract. HPTLC was used with the objective of producing a chromatogram (phenolic fingerprint) of both the extracts to understand whether any additional peaks are obtained or not. The purpose of HPTLC was only semi-quantitative to generate the total cumulative AUC. It is not possible to separate 17 phenolic compounds using a single mobile phase in HPTLC. Henceforth, comparison of chromatogram of the extracts was the only objective behind doing HPTLC. Rutin, Gallic acid & Quercetin could be identified because external standards were available to us. Table 1 indicates the phenolic profiling data obtained from the extract produced from MHG and that of Soxhlet extraction. Results are clearly indicative of the fact that the extract produced from MHG is significantly richer in phenolic principles when compared to the extract obtained from Soxhlet extraction. The quality of the extract is governed by the quantity of phytoconstituents present in it. In this regard richness of the extract in terms of phenolic principles produced from MHG extraction is a clear indicator of its superiority over its Soxhlet counterpart.

Table 1. Phenolic profiling for extract quality comparison (optimal MHG vs. Soxhlet)

Compound	Soxhlet (mg/g of dried extract)	MHG (mg/g of dried extract)	% increase or decrease (MHG)
Benzoic acid	0.40	0.35	↓ 12.5%
p-hydroxy benzoic acid	0.05	0.01	↓ 80%
Salicylic acid	0.27	0.33	↑ 22.22%
3-hydroxy benzoic acid	0.07	0.01	↓ 85.71%
t-cinnamic acid	0.81	2.76	↑ 240.74%
2,4-dihydroxybenzoic acid	0.25	0.02	↓ 115%
Gentisic acid	0.16	0.24	↑ 50%
Protocatechuic acid	0.07	0.07	=
p-coumaric acid	0.09	0.45	↑ 400%
o-coumaric acid	0.03	0.05	↑ 66.66%
Vanillic acid	0.04	0.09	↑ 125%
Gallic acid	0.14	0.65	↑ 364.28%
Caffeic acid	0.46	19.88	↑ 4,221.73%
Ferulic acid	0.31	0.49	↑ 58.064%
Sinapic acid	0.15	0.22	↑ 46.66%
Ellagic acid	0.01	0.02	↑ 100%
Chlorogenic acid	0.01	0.02	↑ 100%

3.3 *HPTLC analysis*

The purpose of doing HPTLC was to compare the chromatograms of extracts obtained from MHG optimal and Soxhlet (in terms of cumulative AUC and no. of peaks). The quality of the extract depends on the number of phytoconstituent present, which has already been ascertain through phenolic profiling using LC-MS/MS as explained in the above section. HPTLC is a second line confirmation of the quality by comparison of chromatograms. Chromatograms of the extract obtained from the optimal MHG protocol and Soxhlet were compared. Results indicate that the cumulative AUC for the chromatogram obtained from the extract produced from the MHG protocol was 110% more than that of the Soxhlet extract (Cumulative AUC of extract obtained from MHG was found to be 21,512.1 ± 957.84, and that obtained from Soxhlet was found to be 10,200.5 ± 470.81) (Figures 7 and 8). The chromatogram obtained from the extract produced from the highest power level of 510 W showed the presence of 1 peak with an AUC of 3,376.5 ± 169.08, indicating massive degradation of other phenolic principles (Figure 9). This observation is in absolute agreement with the findings of TPC, which showed a remarkable decline at 510 W. MHG extract showed the presence of rutin (Rf = 0.01 and AUC = 3,998.3 ± 148.7), gallic acid (Rf = 0.48 and AUC = 4,138.4 ± 159.3), and quercetin (Rf = 0.57 and AUC = 997.3 ± 84.7), which were identified using an external standard. On the other hand, the extract obtained from highest microwave power (510 W) showed the presence of only rutin with cumulative AUC of 3,376.5 ± 169.08, indicating complete thermal degradation of the other principles. The phenomenon can be understood in light of the statement; different compounds are sensitized differently towards microwave heating and are likely to undergo thermal degradation accordingly. The pattern of chromatogram for MHG extract and Soxhlet extract was very similar with only difference in AUC. This fact indicates that no undesirable adduct was formed due to microwave exposure. Despite the fact that chromatogram pattern was similar for both the extraction methods but the cumulative AUC was significantly higher in the case of the extract obtained from MHG, indicating better quality of extract.

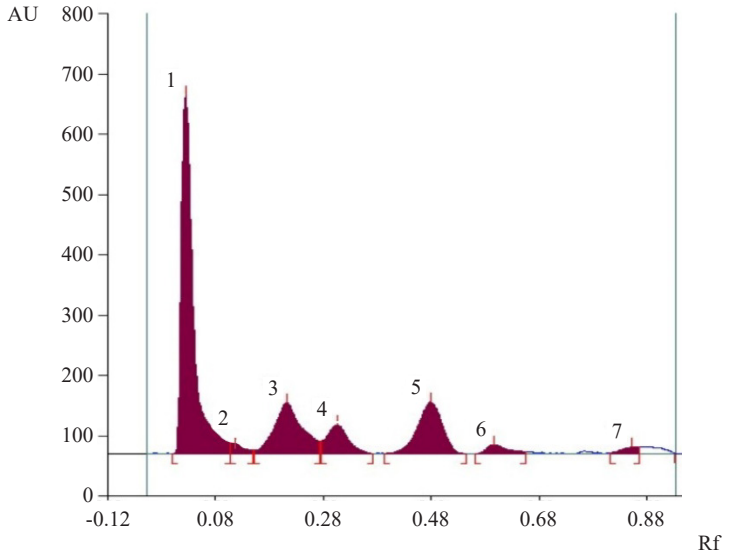


Figure 7. HPTLC chromatogram of L. sativa extract obtained at 340 W during MHG (cumulative AUC = 21,512.1 ± 957.84) (1. Rutin, 5. Gallic acid and 6. Quercetin)

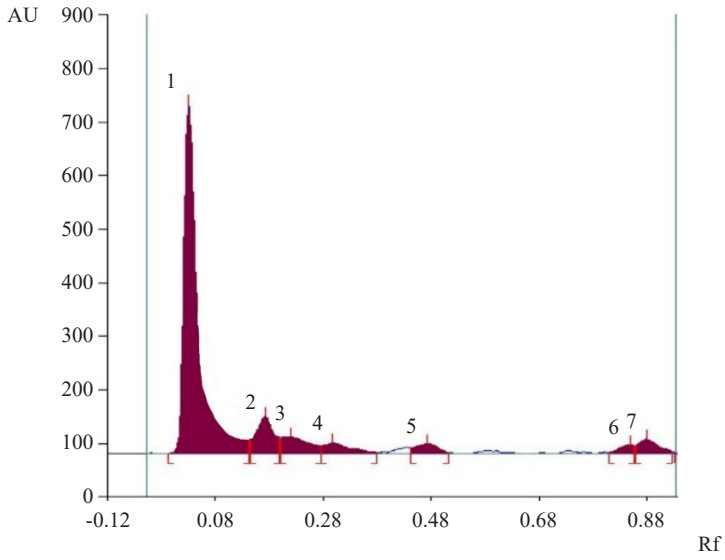


Figure 8. HPTLC chromatogram of L. sativa extract obtained from Soxhlet extraction (cumulative AUC = 10,200.5 ± 470.81) (1. Rutin and 5. Gallic acid)

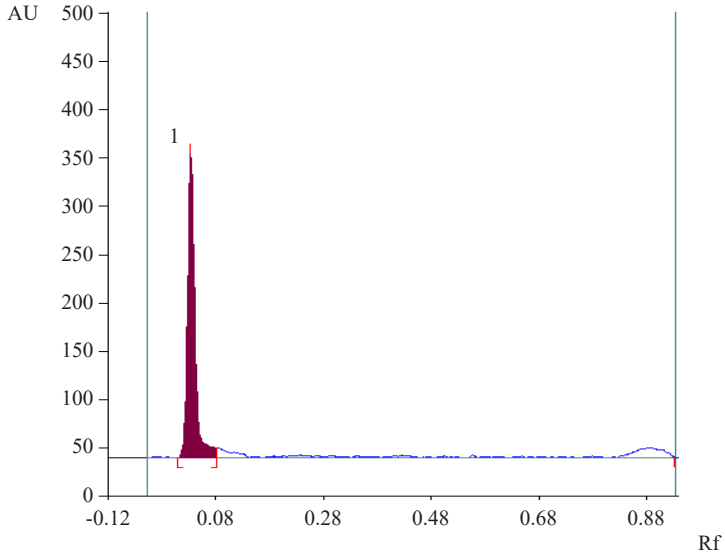


Figure 9. HPTLC chromatogram of L. sativa extract obtained at 510 W during MHG (cumulative AUC = 3,376.5 ± 169.08) (1. Rutin)

3.4 *Dot-blot analysis (TLC bioautography)*

It is a method for real-time detection of antioxidant compounds on the TLC plate itself. Using a microwave for the purpose of extraction can lead to doubts regarding the formation of undesirable by-products or compromised biological activity. In order to crush such doubts, antioxidant activity was chosen as the indicator of the biological potential of the biomass. It can be anticipated that the antioxidant activity of the extract, which is symbolic of the extract of being bioactive in nature, is retained then other activities can also be considered to be intact.

In the case of dot-blot analysis, the antioxidant active zones are reflected as yellow segments in a purple background. The purple background is due to staining by the DPPH reagent. The AUC of total active zones for the extract obtained from 340 W microwave power was found to be 48.23% (AUC = 42,374.5 ± 2,747.6) more than the

AUC obtained from the extract produced from Soxhlet (Figure 10 and Figure 11). Phenolic and flavonoid compounds are majorly responsible for antioxidant activity.

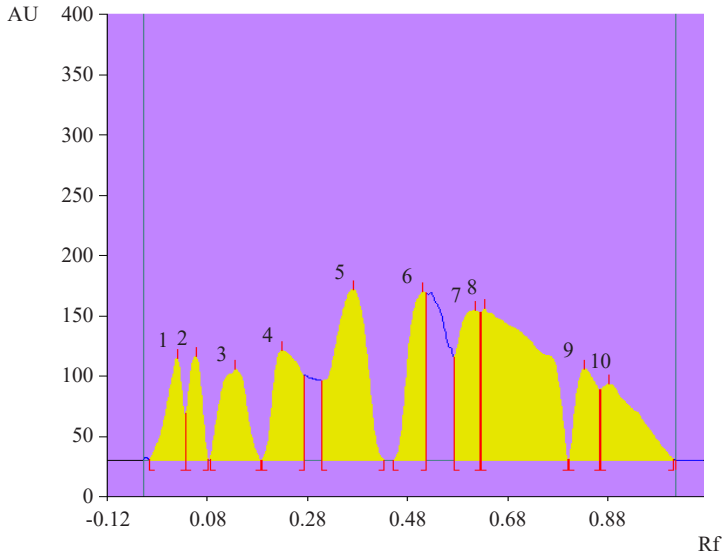


Figure 10. Chromatogram of TLC dot-blot representing antioxidant active zones of L. sativum extract from optimal MHG extraction (cumulative AUC = 42,374.5 ± 2,747.6)

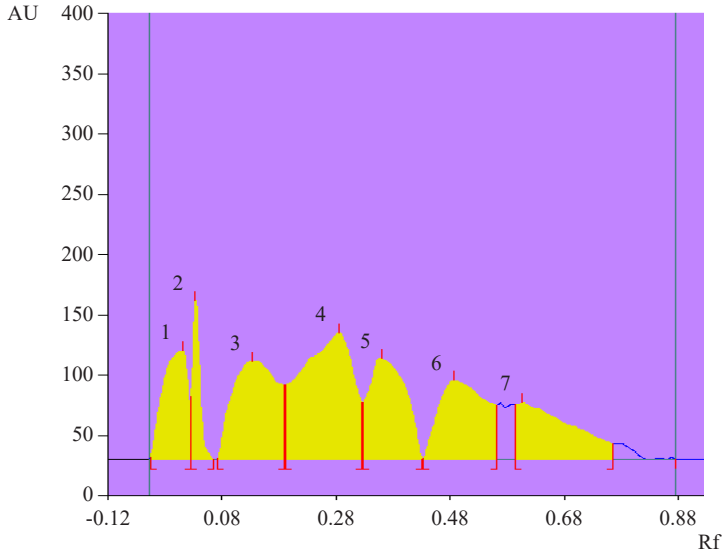


Figure 11. Chromatogram of TLC dot-blot representing antioxidant active zones of L. sativum extract from Soxhlet extraction (cumulative AUC = 28,586.6 ± 1,739.4)

The HPTLC chromatogram (phenolic fingerprinting) of the extract produce from 340 W microwave power level showed the presence of peaks with larger AUC values & have also exhibited improved antioxidant activity when compared to the extract produce from Soxhlet. The above findings indicate that the extract produce from MHG not only has a greater number of phytoconstituents but also is capable of exhibiting improved antioxidant activity when compared to the extract obtained from Soxhlet.

3.5 *SEM*

SEM of leftover biomass after MHG extraction was done to investigate the surface morphology changes that occurred during the extraction process. The SEM examination was conducted at 500x magnification level to evaluate surface morphological changes. Figure 12 demonstrated extensive surface disorientation and distortion in samples subjected to the MHG protocol when compared with sample from Soxhlet extraction.

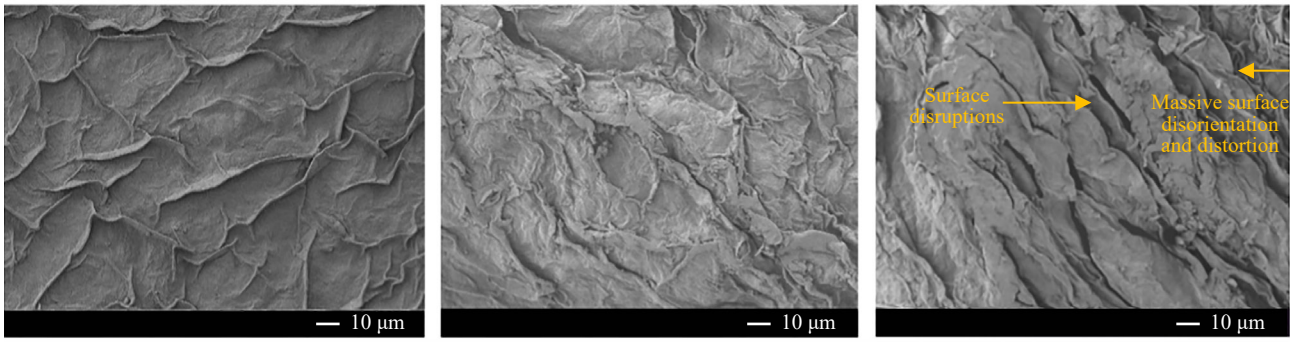


Figure 12. SEM micrographs representing surface morphological changes of biomass during extraction (a) Control, (b) Optimum MHG and (c) Soxhlet extraction surface disorientation and distortion

Traditional extraction methods generally involve three sequential stages: solvent penetration through the cell wall, target analyte dissolution, and direct diffusion of dissolved compounds through internal cellular pathways (Kala et al., 2016). However, in MHG extraction, the penetration and dissolution phases are bypassed as no extraction solvent is utilized in this process. The surface morphology and cellular disruption result from localized thermal stress and internal pressure accumulation caused by the vaporization of internal moisture content within the plant material (Farhat et al., 2010; Kusuma et al., 2018; López-Hortas et al., 2020; Singh Chouhan et al., 2019). Thus, a direct exit channel is created for the diffusion of the analytes from the inside to the external plant surface. Moreover, in the case of MHG, the movement of heat and mass takes place from inside the plant matrix to the outside bulk. Since both the movements are unidirectional hence, the overall extraction process is accelerated. Whereas, in the case of Soxhlet extraction, mass transfer as usual is always from indie of the plant matrix to the outside bulk, whereas heat transfer takes place from outside bulk to the inside of the plant matrix. Since both movements are opposite to each other, the overall process is slowed down.

3.6 *Energy consumption, environmental ecology and production cost*

Energy consumption for MHG (optimal) extraction and Soxhlet extraction was calculated in kilowatt-hours (kWh) using the methodology described by Kusuma and Mahfud (2017), with these values subsequently used to assess carbon emissions. Based on the standard emission factor of 800 g CO_2 per unit of fossil fuel combustion, the environmental impact analysis revealed substantially lower CO_2 emissions for MHG extraction (142.85 g CO_2/g of extract) compared to Soxhlet extraction (7,024.39 g CO_2/g of extract). It should be noted that this assessment considers only CO_2 emissions and excludes other greenhouse gas contributions (Farhat et al., 2009). Production cost was calculated by referring to the industrial electricity tariff of the state power supplier (Chhattisgarh State Power Distribution Company Limited) for the financial year 2024-25. The costing for per g extract production from MHG was found to be ₹ 1.21, whereas the costing for per g extract production from Soxhlet extraction was found to be ₹ 59.71.

4. Conclusion

The results of the MHG protocol are clearly indicative of its superiority in terms of yield and quality over its Soxhlet counterpart. The final optimal extraction condition for the MHG protocol turned out to be extraction at 340 W power level operated for 4 cycles of 5 min each. In order to ensure judicious utilisation of generated waste, the leftover biomass was converted to compost by sending the sample to the municipal compost centre situated in Dipka Korba (C.G). The obtained compost was used in the medicinal plant garden of the Department as a source of biofertilizer.

With time, it is very important that we adopt to the changing demands of the global community and environment. Extract obtained from such an innovative method provides a substantial advantage over conventional approaches by eliminating the need for organic solvents, reducing process time, and maintaining the integrity of the polyphenolic compound while achieving higher yield. This extract can be incorporated into functional foods, nutraceuticals, and dietary supplements, creating an opportunity for developing high-quality food products. The elimination of solvent is particularly beneficial for formulation related with cosmetics, providing an eco-friendly, chemical free product reducing the risk of skin irritation and improving product safety. With so much discourse going on in terms of reducing carbon emission, such ecofriendly techniques shall lead the way in the near future. Adoption of MHG may take some time while more knowledge builds up. Table 2 describes a consolidated performance comparison of Soxhlet extraction and MHG (optimal).

Table 2. Performance comparison of Soxhlet extraction and MHG (optimal)

Parameters		Methods	
		Soxhlet Extraction	MHG (optimal)
Performance comparison	Extraction time (in min)	1,440 min	28 min (inclusion of cooling time)
	Extraction yield	0.82 ± 0.05%	1.12 ± 0.02%
Phenolic content comparison	TPC of dried extract (mg GAE/g of dried extract)	496.36 ± 54.27	3,436.5 ± 47.32
	Cumulative AUC (HPTLC chromatogram)	10,200.5 ± 470.81	21,512.1 ± 957.84
	Phenolic profiling (LC-MS/MS)	17 phenolics identified in which 4 phenolics (benzoic acid, p-hydroxy benzoic acid, 3-hydroxy benzoic acid and 2,4-dihydroxybenzoic acid) were found in increased concentration compared to MHG (optimal)	17 phenolics identified in which 12 phenolics (salicylic acid, t-cinnamic acid, gentisic acid, p-coumaric acid, o-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, sinapic acid, ellagic acid and chlorogenic acid) were found in increased concentration compared to Soxhlet extract
	Cumulative AUC of antioxidant zone	28,586.6 ± 1,739.4	42,374.5 ± 2,747.6
Energy audit	Total energy consumed	3.6 KWh	0.1 KWh
	Relative CO_2 emission	7,024.39 g of CO_2 per g of extract	142.85 g of CO_2 per g of extract
	Production cost	₹ 59.71 per g of extract	₹ 1.21 per g of extract

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Conflict of interest

The authors declare no competing financial interests

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