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Extraction, characterization and optimization of phenolic compounds from Siam weed (*Chromolaena odorata*)

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Abstract

In this study, methanol and water were used as solvents to extract the phenolic compounds from Siam weed. The qualitative and quantitative analyses of the extract were performed to determine the suitability of the weed in the treatment of certain diseases. The process variables for the extraction were optimized. The results show that methanol produced higher quantity of bioactive components than water. The qualitative analysis show that Siam weed contains many bioactive compounds. The quantitative results show that it contains phenolic 52.3 Ggae/100 g, Tannin 12.3%, Alkaloids 5.5 %, Steroids 0.6%, Flavonoids 0.6 % and Glycosides 0.3 %. The optimum yield of 52.32Ggae/100g phenolic compound is obtained at Time of 1.88 hrs. Temp. 45 °C and Dosage 2.50 g.

Keywords: Extraction; Optimization; Siam weed; Bioactive; Solvent

1. Introduction

With the increasing demand for herbal medicinal products for primary healthcare worldwide, there is urgent need for medicinal plant extract manufacturers and essential oil producers to use the most appropriate extraction techniques [1]. Extraction of medicinal plants is a process of separating active plant materials or secondary metabolites from inert material using an appropriate solvent and standard extraction procedure. Several methods were used in the extraction of medicinal plants such as maceration, infusion, decoction, percolation, digestion, Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extraction. In addition, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC) were used in the separation and purification of the secondary metabolites [2].

Maceration is a solid-liquid extraction process in which coarsely powdered drug material, such as leaves, stem bark, or root bark, is placed within a container, and menstruum is poured on top until the drug material is completely covered for at least three days [3]. Infusion is the soaking or immersing the plant parts to be used in boiling water for 15 minutes before filtering through filter paper [4]. This is an extraction procedure created by boiling the plant material with water to the extent that the volume is reduced to one-fourth of its original size after the process [5]. Percolation is the utilization of a percolator for the extraction of active substances by the creation of tinctures and fluid extracts [6]. Soxhlet extraction is a continuous solid/liquid extraction or a continuous heat extraction carried out in a glass equipment known as a Soxhlet extractor [7]. Supercritical fluid extraction is a novel extraction in which a component is extracted from a matrix using a supercritical fluid as solvent. . Microwave-assisted extraction works on the principle in which material absorbs electromagnetic waves and converts them to heat energy that is used for extraction [8]. Ultrasound-assisted extraction (UAE) or sonication extraction employs ultrasound with frequencies ranging from 20 KHz to 2000 KHz, which improves cell permeability and causes cavitation improves surface contact between solvents and samples as well as cell wall permeability thus, enabling compound release and increasing mass transport of the

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solvents into the plant cells [9] In all the above processes solvent extraction is adjudged as the best because of its adaptability for a continuous process operation, high extraction efficiency, wide range of application, high profit and yield [10].

Phenolic compounds are organic compounds produced by any living thing like microorganisms and plants that are not directly involved in the normal growth, development or reproduction of the organism. These substances are generally called secondary metabolites [11]. They benefit their producers by acting as poisons in other to protect them against competitors, predators or parasites .Other secondary metabolites in plants are alkaloids, terpenes, Nitrogen-containing compounds, Lipids, Organo-sulfur compounds and Carbohydrates [12]. Phenolics compounds are further classified into Simple phenolic, Tannins, Coumarins, Flavounoids, Chromones and Xanthones, Stilbenes and Lignans [13] A wide spectrum of bioactivities is exhibited by these compounds such as anti-inflammatory, immunostimulatory, anticancer, antioxidant, antimicrobial [14].

Siam weed scientifically known as *chromonela odorata* leaves came to Nigeria in 1960 and are traditionally used to cure blood pressure, heals cyst, cancer and diabetes [15]

This research is aimed at extraction, characterization and optimization of phenolic compounds using response surface methodology (RSM). The experimental data are evaluated to fit a statistical model. The coefficients of the model are represented by constant term, A, B and C (linear coefficients for independent variables), AB, AC and BC (interactive term coefficient), A^2, B^2 and C^2 (quadratic term coefficient). Correlation coefficient (R^2), adjusted determination coefficient (Adj-R^2) and adequate precision are used to check the model adequacies; the model is adequate when its P value < 0.05, lack of fit P value > 0.05, R^2 > 0.9 and Adeq Precision >4 [16,17]. Differences between means can be tested for statistical significance using analysis of variance (ANOVA) [18].

2. Materials and Methods

The materials used in this investigation include : Siam leaves, HCl, Wagner reagent, iodine crystal, potassium iodide, Ferric chloride, Conc. sulphuric acid, NaOH, Dinitro salicylic acid (DNS), Distilled water, UV Visible Spectrophotometer(Ultraspec2100 pro), Electric weighing balance(YP502N NO:SHP1100313200), Water bath (DK600 Gallenkomp England) , Laboratory drying oven(DHG-9101-ISA), Bunsen burner , volumetric flasks , measuring sylinders and petridishes, reflux condenser sieving net, wooden motar, methanol, Methanol, Hexane, distilled water, sodium hydroxide (NaOH), Hydrochloric acid, Magnesium chloride ($Mgcl_2$), sodium ethanoate (CH_2COON_a), DTPA- Diethylene-tri-amine pent acetic acid, Triethanolamine (TEA), Potassium Dichromate ($K_2Cr_2O_7$), Ferrous Ammonium sulphate ($Fe(NH_2)_2$ (Sp): $6H_2O$ phosphoric acid(H_3PO_4), Sodium fluoride(NaF), Diphenyl amine ($C_6H_5NH_6H_5$).

2.1 Extraction Procedure

- **Sample preparation:** The leave samples were obtained from Obollo-Afor in Udenu Local Government Area of Enugu State Nigeria and it was authenticated at the Botany department of the University of Nigeria. The samples were air dried for 2 days, ground with wooden mortar and sieved to 250µm.
- **Leave Extraction:** The extraction was carried out using Soxhlet apparatus as described by [19] 100 ml of methanol or water was added into the two-neck round bottom flask. Then 10 g of the ground sample was wrapped in a filter paper. The paper was sealed with office pin to avoid direct contact of the sample and the solvent. The wrapped sample was introduced into the chamber of the Soxhlet apparatus, and the column was connected to the round bottom flask before mounting to a heating mantle. The cooling water was connected through the condenser. The extraction took place for about 2 hours, making over 5 cycle extraction.
- Qualitative phytochemical analysis of aqueous extracts of Siam plant leave was carried out on the extract using standard procedure to identify the constituents as described by [20, 21]

2.2 Test for Tannins

1ml of extract was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and green or a blue – black coloration was observed which confirms the presence of tannin.

2.3 Test for Saponins

About 5ml of the extract was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of

olive oil and shaken vigorously, then observed for the formation of emulsion which confirms a positive presence of Saponins.

2.4 Test of Flavonoids

3ml of 1% Aluminum chloride solution were added to 5ml of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5ml of dilute ammonia solution were added to the above mixture followed by addition of concentrated H_2SO_4. A yellow coloration disappeared on standing. The yellow coloration which disappeared on standing indicates a positive test for flavonoids.

2.5 Test for Steroids

2ml of acetic anhydride was added to 2 ml extract of each sample followed by careful addition of 2ml H2SO4. The color changed from violet to blue or green indicate the presence of steroids.

2.6 Test for Terpenoids (Salkowski's test)

Five ml of each extract was mixed with 2ml of chloroform, and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

2.7 Test for Cardiac Glycosides and Cardenolides (Keller – Killani test)

5 ml of each extracts was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of cardenolides which confirms a positive presence of cardenolides. A violet-green ring appearing below the brown ring, in the acetic acid layer, indicates the positive presence of glycoside.

2.8 Alkaloids

1ml of the extract was stirred with 5ml of 1% aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1ml of the filtrate was treated with a few drops of either Mayer's reagent (Potassium mercuric iodide- solution) or Wagner's reagent (solution of iodine in Potassium iodide) or Dragendorff's reagent (solution of Potassium bismuth iodide). The formation of a cream color with Mayer's reagent and reddish-brown precipitate with Wagner's and Dragendorff's reagent give a positive test for alkaloids.

2.9 Phenol

5ml of the extract was pipetted into a 30 ml test tube, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added and left to react for 30 min. Development of bluish green color was taken as a positive presence of phenol.

2.10 Quantitative phytochemical analysis

The phytochemicals which are present in the aqueous extracts of Siam plant leaves was determined and quantified by standard procedures

2.10.1 Determination of total phenolic compounds

100 mg of the extract of the sample was weighed accurately and dissolved in 100 ml of triple distilled water (TDW). 1 ml of this solution was transferred to a test tube, then 0.5 ml 2N of the Folin Ciocalteu reagent and 1.5 ml 20% of Na_2CO_3 solution was added and ultimately the volume was made up to 8 ml with TDW followed by vigorous shaking and finally allowed to stand for 2 hours after which the absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of garlic acid [22]

2.10.2 Determination of total flavonoids

The method is based on the formation of the flavonoids - aluminum complex which has an absorptivity maximum at 415 nm. 100 µl of the sample extracts in methanol (10 mg/ml) was mixed with 100 µl of 20% aluminum trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5ml. The absorption at 415 nm was read after 40 minutes. Blank samples were prepared from 100 ml of sample extracts and a drop of acetic acid, and then diluted to 5ml with methanol. The absorption of standard solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates [23]

2.10.3 Determination of total alkaloids:

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle until the precipitation was completed, the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed [24]

2.10.4 Determination of total tannins

500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.I N HCl and 0.008 M potassium Ferro cyanide. The absorbance was measured at 120 nm within 10 min [23].

2.10.5 Determination of total Saponins

The samples were ground and 20 g of each were put into a conical flask and 100 cm3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55 ℃. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 ℃. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated. [23]

2.10.6 Determination of Cardiac Glycosides

Cyanogenic glycoside quantitative determination methodology used in this research is that by Amadi et al. [12] as reported by Ejikeme et al. [24]. It was weighed into a 250 cm3 round bottom flask and about 200 cm3 of distilled water was added to one gram of each dry wood powder sample and allowed to stand for 2 hours for autolysis to occur. Full distillation was carried out in a 250 cm3 conical flask containing 20 cm^3 of 2.5 % NaOH (sodium hydroxide) in the sample after adding an antifoaming agent (tannic acid). Cyanogenic glycoside (100 cm3), 8 cm3 of 6 M NH_4OH (ammonium hydroxide), and 2 cm3 of 5 % KI (potassium iodide) were added to the distillate(s), mixed, and titrated with 0.02 M $AgNO_3$ (silver nitrate) using a microburette against a black background. Turbidity which was continuous indicates the end point. Content of cyanogenic glycoside in the sample was calculated as Cyanogenic glycoside (mg 100 g) = Titre Value (Cm3) × 1.08 × exact volume Aliquot volume (Cm3) × sample weight(g) × 100.

3. Results and Discussions

Table 1 The Phytochemical Screening of Siam leaf extracts

Parameter	Sample	Result
Alkaloid	Methanol Extract	++++
	Water extract	-
Tannins	Methanol Extract	++++
	Water extract	++
Steroids	Methanol Extract	++++
	Water extract	+++
Flavonoids	Methanol Extract	++++
	Water extract	++
Phenols	Methanol Extract	+++
	Water extract	-

Saponins	Methanol Extract	+++
	Water extract	+
Glycosides	Methanol Extract	++++
	Water extract	++

Code: ++++ (very much present), +++ (much present), ++ (Present), + (slightly present), -(absent)

The weed extract showed a better result with methanol solvent than water for the seven phytochemical tests (Table 1). This is because methanol is volatile and permeates into the cells and solubilizes the bioactive materials thus creating better surface for mas transfer. A similar result was obtained by [17]. Phytochemical compounds such as alkaloids, saponin, tannin, phenolic compounds, flavonoids, glycosides, steroids, and flavonoid are very much present {+++} with methanol solvent while water solvent they only showed present and slightly present. Furthermore, only the extract with methanol solvent was subjected to further analytical tests for the quantification of phytochemical compounds (Table 2).

Table 2 Quantitative compositions of Siam weed

Bioactive-active components	**Quantity**
Phenolic	52.3 Ggae/100 g
Alkaloids	2.6 %
Steroids	0.6 %
Flavonoids	32.6%
Glycosides	753 mg/100 g
Tannins Saponin	72 mg/100 g 25.4 %

The quantitative result shows that Siam weed contain mainly phenolic compounds, Glycosides, Saponnin and Flavonoids with values 52.3 Gae/100 g,753 mg/100 g, 25,4 % and 32.6 % respectively. Phenolic are antioxidants in human and plants [25]. It has the potential for amelioration of diseases by improving the dietary intake of nutrients with antioxidant properties, such as vitamin E, vitamin C, β-carotene, and carotenoids [26]. Cardiac glycoside has been used in treatment of congestive heart failure due to its direct action which increases the force of myocardial contraction. The weed also contain high quantity of Flavonoids which are known to have antioxidant effects and have been shown to inhibit the initiation, promotion, and progression of tumors and reduction of coronary heart disease [27]. Apart from the antioxidant properties of flavonoid, it possesses other biological functions such as the protection against platelet aggregation, microorganisms, hepatotoxins, viruses, tumors, ulcers, free radicals, inflammation, and allergies [28].

Saponins protects microbial attack in plants and very useful in treating yeast and fungal infections [1]. Alkaloids have equally been exploited for their importance in traditional pharmaceutical usage.

Table 3 RSM Result for the extraction of phenolic compounds

	Design for Siam Weed				
Run	**Std**	**A:Time(hour)**	**B:Temperature(°C)**	**Dosage (g)**	**Phenolic Ggae/100g**
3	1	1	60	2.5	27.42
1	2	1	30	2.5	47.21
14	3	3.5	45	2.5	52.33
4	4	6	60	2.5	23.63
11	5	3.5	30	4	28.24
6	6	6	45	1	50.05

17	7	3.5	45	2.5	23.63
12	8	3.5	60	4	21.42
7	9	1	45	4	30.42
10	10	3.5	60	1	48.66
5	11	1	45	1	33.26
8	12	6	45	4	26.42
2	13	6	30	2.5	37.16
15	14	3.5	45	2.5	52.30
13	15	3.5	45	2.5	52.33
16	16	3.5	45	2.5	52.33
9	17	3.5	30	1	38.42

Table 4 ANOVA

Analysis of variance table [Partial sum of squares]							
	Sum of		**Mean**	**F**			
Source	**Squares**	**DF**	**Square**	**Value**	**Prob > F**		
Model	1958.756	9	217.6396	5.584089	0.0168	significant	
A	0.094612	1	0.094612	0.002428	0.0021		
B	113.1008	1	113.1008	2.901884	0.0023		
C	510.2415	1	510.2415	13.09152	0.0085		
A2	328.3459	1	328.3459	8.424536	0.0229		
B2	394.8014	1	394.8014	10.12962	0.0154		
C2	301.0514	1	301.0514	7.724228	0.0273		
AB	10.3684	1	10.3684	0.266027	0.0019		
AC	108.056	1	108.056	2.772448	0.0098		
BC	72.7609	1	72.7609	1.866863	0.0041		
Residual	272.8246	7	38.97495				
Lack of Fit	272.8239	3	90.94131	505229.5	< 0.0001	significant	
Pure Error	0.00072	4	0.00018				
Cor Total	2231.581	16					

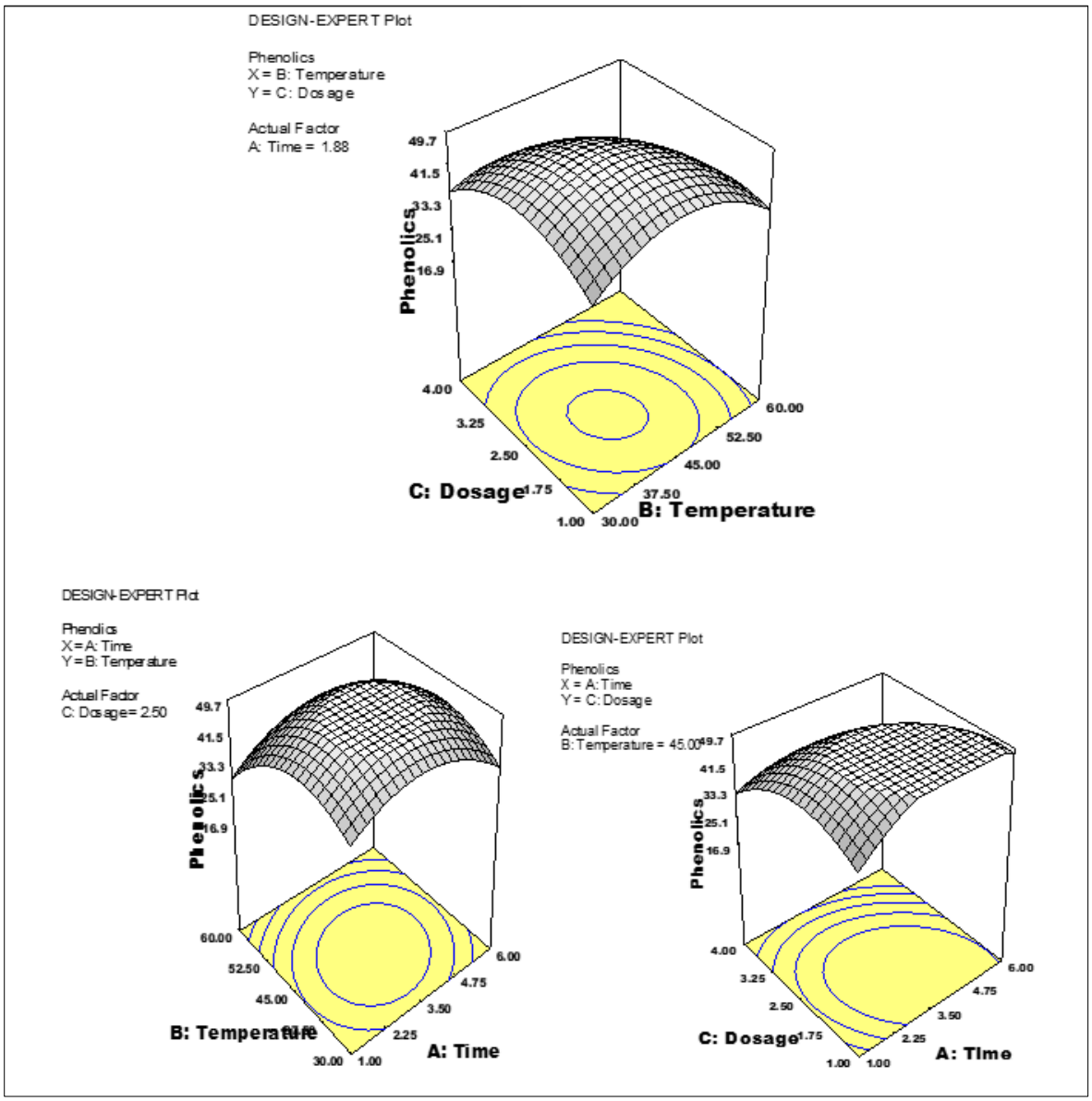

Figure 1 3-D Plots for the effect of process variables on the extraction of phenolic compound

In this study, the Design Expert 12 software (Trial version, Stat-Ease Inc) was used to analyze the response surface. First, a Box–Behnken experiment design was selected to establish a second order polynomial model of extraction of phenols from Siam weed. Indeed, this design is easy to implement compared with other optimization methods due to time saving by reducing the number of experiments. One response the total phenolic compound was studied: responses were evaluated as a function of the combined effect of three parameters i.e.,: Time (A), Temperature (B), and Dosage (C), each one being studied at three levels- low, middle and high.. The variables and experimental design levels used in the Box-Behnken design are reported in Table 3. On total, the Box-Behnken matrix was designed with these three independent variables (Table 2) and involved 15 experiments, including a triplicate at the central point. [Table 2]

A second order polynomial model equation was used to fit the generalized form of mathematical quadratic response surface. The model can be expressed by the following equation (1).

$$\text{Phenolic} = 52.324 - 0.10875\,A - 3.76\,B - 7.986\,C - 8.830\,A^2 - 9.683\,B^2 - 8.45575\,C^2 + 1.61\,AB - 5.1975\,AC - 4.36\,BC \quad (1)$$

Where A is Time, B is Temp and C is dosage. Analysis of variance was performed to test the significance and adequacy of model. The adequacy of the model was assessed using the R-squared values, the coefficient of determination (<), the adjusted R-squared (AB5 <), and the probability value as well. P-values ≤ 0.05 were considered statistically significant.

The model F value is high 5.58 significant and R^2 of 0.877 is adequate. The optimum yield of 52.32Ggae/100g phenolic compound is obtained at time of 1.88hrs, temperature of 45 ^{0}C and Dosage of 2.50 g. The 3-D response surface plots shown in fig. 1 represents an interactive effects of the variables and the elliptical nature of the graph depicts indicates a good interaction of the variables.

4. Conclusion

This study indicates that higher quantities of the bioactive compounds are produced with methanol than water solvent. The qualitative analysis reveals that there are more phenolic compounds than other bioactive compounds in the weed. The optimum yield of 52.32 Ggae/100 g phenolic compounds is obtained at a time of 1.88hrs, temperature of 45^{o}C and Dosage of 2.50 g.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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