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Optimization of Phenolic Compounds Extraction from *Ruta Chalepensis* (Tenadam) Leaves and its Antioxidant Activity

Gebeyanesh Endalkachew Wondafrash*

Department of Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

Abstract

Phenolic compounds extraction is a great research required to their bioactive properties such as antioxidant, antimicrobial, anti-mutagenic, anti-viral and anti-inflammatory activity. In this study extraction, optimization and determination of phenolic compounds from *Ruta Chalepensis* (Tenadam) leaves has been recognized as good sources for antioxidant potential on butter. The phenolic compounds were extracted by soxhlet apparatus (Scintifica VELP modern soxhlet) and the extraction process parameters such as extraction time (90-180minute), type of solvents (ethanol, methanol and acetone) and solid mass to solvent ratio (0.05-0.10) were optimized by using response surface methods. Results showed that extraction conditions significantly ($P<0.05$) affect phenolic compounds of *Ruta Chalepensis* leaves extract. The yield of phenolic compounds was ranged from 150.09 mg GAE /gm of dry weight to 334.12mgGAE /gm of dry weigh. The optimal extraction conditions predicted by the models were time at 105.34 minute, ratio at 0.06, and solvent by Acetone gave the optimal predicted values of 334.22 mg GAE/gm of dry sample. Under optimized conditions the experimental values were agreed with the values predicted by the proposed models. Antioxidant from natural origin has attracted to protect the human body from free radicals, due to its high content of phenolic compounds. The ability of phenolic antioxidant was analyzed in vitro by DPPH, ferric reducing power assay (frap). The IC50 which is concentration required to quench 50% of the DPPH radical, was computed to be 17.55 mM of ascorbic acid and 37.156 mM of extract of *Ruta Chalepensis* leaves respectively. And the smaller the value of the IC50 indicates the higher antioxidant activity. The ferric reducing value was 115.75mgAAE per gram of dry weight and the reducing value was significant indicator of its potential antioxidant activity. *Ruta Chalepensis* leaves have flavonoid content that was conducted 36.818 mg QE/gram of dry weight. The extract of *Ruta Chalepensis* leaves (Tenadam) was conducted different functional groups some of them were aromatic, aliphatic, alcohol and hydroxyl compound (phenolic compound), carboxylic compounds and soon.

Keywords: Total phenolic compounds • Antioxidant activity • Optimization • Response surface methodology • *Ruta Chalepensis* Leaves

Introduction

Because of an increasing worldwide demand for phenolic compounds and their increasing application in food industry, the research of phenolic compound extraction has been growing recently whereas, the current problem is producing safe products that is increasing consumer health needs. In addition, there is a great interest in finding safe antioxidant from natural sources. Many researches approved Ruta species was characterized by the presence of alkaloids, flavonoids, coumarins, phenols, tannins, volatile oil, glycosides, terpene and others as possible bioactive compounds. Ruta is an ornamental, aromatic, culinary and medicinal plant, grown in the wild and can be cultivated in gardens [1]. It belongs to the Rutaceae family and it is a hard, evergreen shrub of up to one-meter height with a characteristic grayish color, yellow flowers and a sharp pleasant odor. In Ethiopian, *Ruta Chalepensis* plant traditionally used to treat many diseases like earache, hemorrhoids, influenza, intestinal disorders and drunk as an anti-implantation and uterotonic medicine. In addition, a decoction of root in an alcoholic drink with hot peppers was taken to treat influenza and a leaves of *Ruta Chalepensis* decoction with tea is taken to treat headache, fever, treat stomach-ache and others. In addition to traditional medicine *Ruta Chalepensis* were used commonly as spice during a traditional Ethiopian coffee and tea ceremony and also, the fruits are used as ingredients of the local “berbere” and “mitten shiro” spice mix or flavor [2].

Phenolic compounds are relevant group of plant secondary metabolites (SM) noticed by the presence of aromatic rings linking one or more hydroxyl groups, and regroups numerous groups such flavonoids, anthocyanins, flavanols, flavonols, flavanones, phenolic acids, stilbenes and complex molecules derived from them. Phenolic compounds were spread in the plant kingdom and the richest secondary metabolites of plants, with more than 8,000 phenolic structures currently known. Phenolic compounds extracted from natural sources are an important category of antioxidants and have a variety of physiological activity such as antioxidant, ant mutagenic, antiallergenic, antiinflammatory, ant-diabetic and antimicrobial effects and thus are widely used in the pharmaceutical, nutraceutical and food fields. Antioxidant compound is an important role due to favorable effect on human health and food consumption by reducing the risk of human disease such as, cancer, diabetes, other aging disease. Antioxidant activity presents in plants and play significant role in the protection of cells and organism. the natural antioxidant extracted from plant is useful in medicinally and also possess wide spectrum of biological activities like inflammatory, antibacterial and antiviral activities [3].

Flavonoids are present in most of the plants and are responsible for color of flowers in plants. In human diets, Flavonoids are the richest phenolic which naturally occurring phenolic. They are low molecular weight compounds and containing of fifteen carbon atoms and the C6–C3–C6 structure [4].

Oxidation defeat consumer acceptability of foods by producing low-molecular-weight off-flavor compounds, as well as by damaging essential nutrients. It produces toxic compounds and polymers of lipids and proteins. Oxidation process is the most relevant routs for producing free radicals in food, drugs and even living systems. Free radicals can be causes for many human diseases like cancer, Alzheimer’s disease, cardic reperfusion abnormalities, kidney and liver disease, fibrosis, atherosclerosis, arthritis, neurodegenerative disorders and aging [5]. Due to positive effects on human health, Different methods have been used for extraction of phenolic compounds. Solvent extraction (soxhlet) is one of a process to separate soluble phenolic compounds.

***Address for Correspondence:** Gebeyanesh Endalkachew Wondafrash, Department of Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia, Tel: 251-24031666; E-mail: gebeyae@gmail.com*

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The aim of this study were extract high yield of total phenolic compounds by optimizing the extraction parameters (time, type of solvent and sample to solvent ratio), evaluate total flavonoid content, vitro antioxidant activities determination, and determine antioxidants activity.

Materials and Method

The fresh *Ruta Chalepensis* leaves (Tenadam) were collected from Amhara Region, North Shewa, Debrebrhan, Ethiopia. The leaves were collected from home garden. After collection the leaves were washed by tap water, rinsed by distilled water and shade dried at room temperature for 2 weeks. The dried leaves were crushed in fine powder by a grinding machine and it stored in an airtight container (plastic bag). All Chemicals and reagents used in this research work were methanol (99.8%), ethanol (97%) acetone, Folin-Ciocalteu reagent(FC) reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH)reagent, Gallic acid, quercetin (98%), Sodium carbonate (99.9%), sodium nitrite, aluminum chloride (99%), sodium hydroxide (99.9%), potassium persulfate, potassium ferricyanide (99%), phosphate buffer (PH 6.6), trichloroacetic acid and L-ascorbic acid (99.7%).

Determination of total phenolic compounds

Total phenolic compounds were determined with Folin-Ciocalteu colorimetric reagent, as described before with slight modifications. 1 ml of *Ruta Chalepensis* leaves extract was added in 100 ml volumetric flask and mixed with 5 ml Folin-Ciocalteu reagent. The solution was mixed by manual shaking for 15–20 seconds. After 3 minute, 0.5 ml of saturated sodium carbonate solution (7.5% w/v) was added and the solution diluted to 5 ml with deionized water. The reaction mixture was incubated in dark at room temperature for 30 minute and its absorbance was measured at 765nm against deionized water using UV spectrophotometer (Jasco V-770 spectrophotometer). The total phenolic content was determined using a calibration curve prepared with Gallic acid standard (0.05mg/ml–5mg/ml) as a reference. The value was reported as mg of Gallic acid equivalent (GAE) by reference to Gallic acid standard curve and the results were expressed as milligrams of GAE per gram dry weight of residues (Figure 1).

Antioxidant activity determination

Antioxidant was determined with 2; 2-diphenyl-1-picryl hydroxyl (DPPH) radical scavenging activity, as described before (Diem, Elisa, & Tran-nguyen, 2013) with slightly modification. 3 ml of prepared solution (dissolving 2 mg of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in 100 ml of methanol) were mixed with 1 ml of samples extracts in 100ml volumetric flask then incubated at room temperature for 30 min in the dark place to complete the reaction and the absorbance of the solution was measured at 517 nm using UV-visible spectrophotometer (UV-750). Ascorbic acid was used for the determination reference. The ability of the sample to scavenge DPPH radical was determined from the following equation.

Ferric Reducing Power (FRAP) technique

The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide-ferric chloride method, according to with slight modification; 1 mL of extract was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide in 100 ml volumetric flask. The mixtures were incubated for 20 min, then, 2.5 mL of 10% (w/v) trichloroacetic acid was added. Finally, 2.5 mL of the mixture were taken and mixed with 2.5 mL of distiller water and 0.5 mL of 0.1% iron chloride($FeCl_3$) (w/v) and thoroughly mixed. The intensity of blue green color was measured at 700 nm using UV-Visible spectrometer (UV1800) against a blank. The blank was prepared similarly with above procedure without the sample. Ferric reducing activity was determined as mg ascorbic acid equivalents per gm of dry weight (mgAAE/100 g Dry sample).

Determination of Total Flavonoid Content (TFC)

Total flavonoid content was measured by spectrometrically using aluminum chloride colorimetric assay, with some modification proposed. Briefly, 2ml of extract was added in 50 ml volumetric flask and 0.3ml of 5% (w/v) of sodium nitrite ($NaNO_2$) solution was added and mixed well. The solution was left to rest for 5 minutes. Then, 0.3ml of 10% (w/v) of aluminum chloride ($AlCl_3$) was added and the mixture will be set to rest for 2minute, and 2ml of 1M sodium hydroxide (NaOH) solution was added into the mixture. The solution was form orange color by adding distilled water. The absorbance of Sample was measured

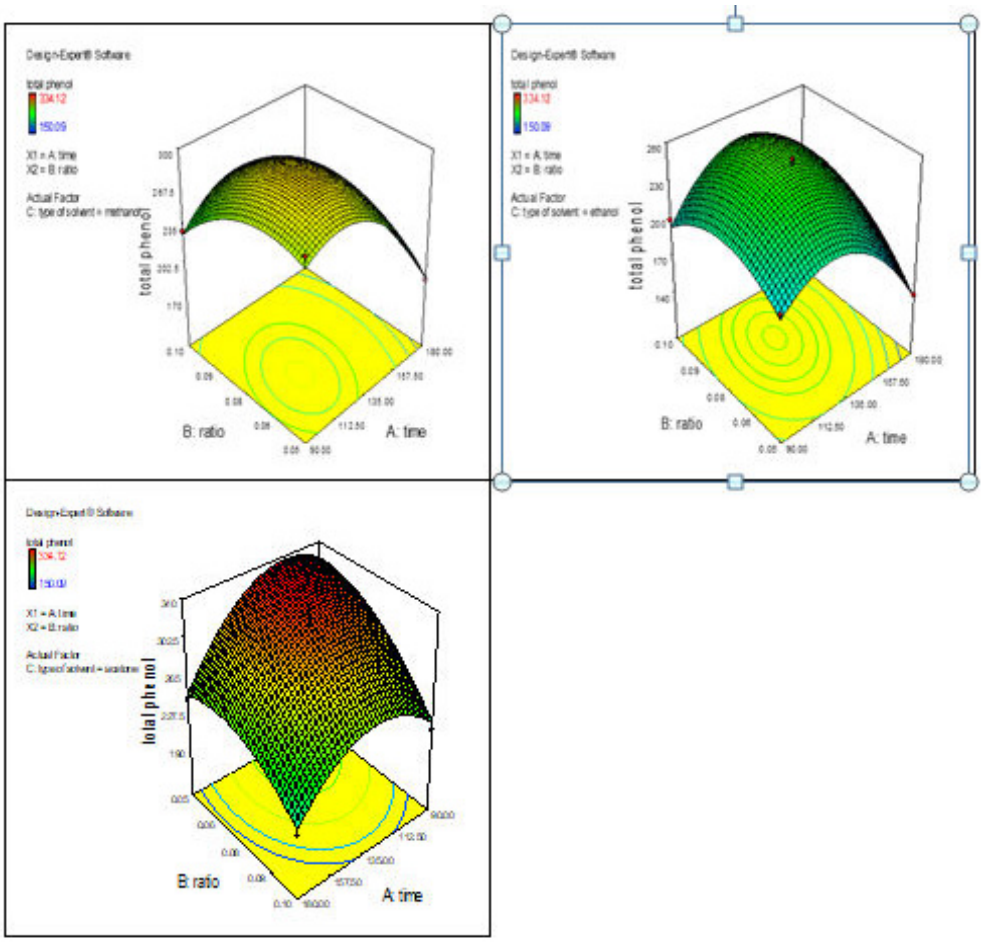

Figure 1. Interaction effect of time and ratio on total phenolic compounds.

at 510 nm against a blank using UV-visible spectrophotometer (UV-750). The blank was prepared the same as the above procedure except sample of extract. The concentration of total flavonoid content was calculated from the calibration plot and expressed as quercetin equivalent (QE) by using the following equation.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

Fourier transform infrared (IS50ABX, FT-IR) spectrometry measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic "fingerprint" of the sample. Small amount of each liquid sample extract was placed directly on the germanium piece of the infrared spectrometer with constant pressure applied. Data of infrared absorbance were collected over the wave number ranged from 4000/cm to 400/cm. All spectra were collected with a resolution of 4.0-1.0 cm and to improve the signal-to-noise ratio. Samples were run in triplicates. The FTIR spectrum of all samples was analyzed on the basis of peak values in the region of infrared radiation.

Evaluation of antioxidant activity by the Rancimat method

Antioxidant activities of extracts obtained from *Ruta Chalepensis* leaves were also measured by the Rancimat method (892 Rancimat apparatus, Metrohm Ltd, Switzerland). Briefly, sample of *ruta Chalepensis* leaves crude extract was added in 3 g of butter with 0.1% concentration (w/w). As the control butter without the extract was run similarly. The maximum level of synthetic antioxidants concentration allowed to be added in food is 0.02% for the safety reasons. In the case of natural antioxidants, higher concentrations (0.05–0.2%) are necessary because of their lower activities and lower toxicity. The concentration of 0.1% was studied as it is most often used in the research as a model substance representing natural antioxidant. The induction period for the formation of oxidative products of oxidizing substrate were measured for antioxidant activity evaluation. A flow of air (20 L/h) was bubbled through the butter heated at temperature of 120°C. All its functions are controlled by the PC connected with RS-232 cable (stabnet 1.1-892 Rancimat software). The test was applied in triplicate. Antioxidant activity of *ruta Chalepensis* leaves was expressed as a protection factor and induction index.

Experimental design and data analysis

Response Surface Methodology (RSM) design was appropriate for determine the effect of the three operating variables of the extraction of phenolic compounds from *Ruta Chalepensis* leaves. These operating variables were extraction time, type of solvent and solid to solvent ratio. The response variable was the extraction yield of phenolic compounds. Central composite designs in which the axial points represent the middle levels. Face centered is desirable for this study because the extraction of phenolic compounds for the location of the optimum point. Three levels, three factors and Each experiment was carried out in triplicate and total of 33 experiments were done. Data was analyzed by analysis of variance, and the mean values were considered significantly different when $p<0.05$. The optimal extraction conditions were estimated through contour and 3D analyses of the three independent variables and each dependent variable.

Results and Discussion

Extraction yield of total phenolic compounds

The curve generated from the plot of absorbance *vs.* concentration should give linear relationship with minimum regression coefficient of 0. 9638. Then the appropriate calibration curve equation is generated in the form of y=0.6972 x-0. 0393. From the equation calculate total phenolic compounds and indicates the yield and run of the experiment. The better extraction yield was obtained at the ratio of 0.08, time of 135 minute and at the type of solvent acetone, at run number (2), the yield was generated 334.12 mg GAE/gm of dry sample. The yield of total phenolic compounds was varied within the range of 150.09 mg GAE/gm of dry sample to 334.12 mg GAE/gm of dry sample.

Experimental design analysis

Analysis of variance (ANOVA) was used to determine the significance of regression coefficients of the model. The coefficient of determination R-squared (R2) indicates the model was accurately representing the relationship between the variables. The model with R2 > 0.75 was considered acceptable. Moreover, the lack of fit need not be significant for a valid model development by response surface method.

According to, a large F value and small p value were an indicative that the independent variables have a significant impact on the respective response variables. The experimental design selected for analysis of variance was response surface methodology (RSM), central composite design (CCD) was selected, specifically, face centered central composite design (FCCCD). RSM was used to investigate the effects of type of solvent, extraction time and solvent ratio on the extraction yield of phenolic compounds. Analysis of variance showed that the selected quadratic models adequately represented the data obtained for total phenolic compounds.

It shows, sum of squares, developed mean square, Adjusted R-square, Predicted R-square, F-value, p-value (prob>F), significance model, and lack of fitness of different models when the response (total phenolic compounds) was evaluated within their respective ANOVA analysis.

Select the highest order polynomial where the additional terms are significant and the model is not aliased. The aim of model fit summary was maximizing the "Adjusted-R square" and the "Predicted-R square". Quadratic model is the significant model represented the data obtained for total phenolic compounds. The quadratic model to have insignificant lack-of-fit because the value of Prob>F is greater than 0.05 and the lack of fit testing was used to verify the adequacy of the fit or the independent variables (extraction parameters) have considerable effect on the yield of total phenolic compound.

A statistical method based on ANOVA was used to obtain the coefficient of determination (R2) for the extraction yields, which were 0.9845, According A good fit with high correlation is achieved if the regression model has an R2 value of above 0.9. The aim of the model is maximizing the "Adjusted R-Squared "and the "Predicted R-Squared". The predicted R-square (Pre. R2) is a measure of how good the model predicts a response value and indicates how well a regression model predicts response values. The value of "Adj R-squared" of 0. 9764 was high and supported a high correlation between the observed and the predicted value of 0. 9682. The high value of" R-squared" for each response indicated that the central composite design fitted well into the quadratic models that were developed.

As illustrated, the model F-value of 77.59 implies the model is significant. There was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant and Values greater than 0.1000 indicate the model terms are not significant. In this case, A, B, C, A2, B2, AB, AC and BC were significant model terms. The "Lack of Fit F-value" of 1.92 and Prob>F of 0.2153implies the Lack of Fit test is not significant. Therefore, the extraction time(A), solid to solvent ratio(B), type of solvent(C) square of extraction time(A2), square of solid to solvent ratio (B2), interaction between ratio and time (AB), interaction between solvent with time (Ac), interaction between solvents with ratio(BC) affect the yield of *Ruta Chalepensis* leave extracts significantly.

Conclusion

This work was schemed at extraction, optimization and determination of antioxidant by in vivo, antioxidant activity on butter and total flavonoid content extracted from *Ruta Chalepensis* leaves.

The extraction was done by soxhlet extractor (scintifica velp). The optimization of phenolic compounds extraction by response surface methodology indicated that type of solvent, sample to solvent ratio and time of extraction are important variables (parameters). The quadratic model selected for optimizing the experimental variables shows that the optimum extraction conditions for total phenolic compound were by solvent type of Acetone, at time of 105.34 minute and ratio of 0.06, which could obtain values of 334.22 mg GAE per gram of dry sample. The antioxidant properties of *Ruta Chalepensis* leaves extract under optimized condition were evaluated for DPPH scavenging activity, and ferric reducing antioxidant potential. The IC50 which is concentration required to quench 50% of the DPPH radical, was computed to be 37.156 mM of extract of *Ruta Chalepensis* leaves. The smaller the value of the IC50 indicates the higher antioxidant activity. The ferric reducing value was 115.75mgAAE per gram of dry weight and the reducing value was significant indicator of its potential antioxidant activity. *Ruta Chalepensis* leaves have flavonoid content that was conducted 36.818 mg QE/gram of dry weight. The extract of *Ruta Chalepensis* leaves (Tenadam) was conducted different functional groups some of them were aromatic, aliphatic, alcohol and hydroxyl compound (phenolic compound), carboxylic compounds and soon.

To conclude, this study was indicated the phenolic compounds extracted from *Ruta Chalepensis* leaves were used for natural antioxidant to lower oxidative instability. Therefore, *Ruta Chalepensis* leaves are a good source of total phenolic compounds and total flavonoid content which possess potent antioxidant activity.

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