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High-Precision On-line Total Phenolic Compounds Analysis Oxidized by Folin Ciocalteu: Application to *Ziziphus* Jujuba Extract

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ABSTRACT

This study deals with a new methodology for total phenolic compounds on-line oxidation utilizing continuous flowing carrier stream technique provided by a system including a micropump, an auto-sampling and a reacting coil. Visible spectrophotometric measurement at 765 nm wavelength was based on fast oxidised phenols by Folin ciocalteu resulting in spectrophotometrically detectable blue chromophores. The reaction was automated to perform rapidity (sampling frequency 11 samples h-1), simplicity, reagents consuming and specificity. It offered a good linearity in a range of 10 - 50 mg mL-1 with no memory effect. It showed excellent precision (RSD lower than 0,4 %) with detection going less than 0,03 ηg.mL-1. The effectiveness of the developed method was verified by its application for the assay of total phenolic compounds in Ziziphus Jujuba fruit extract. This new method can be used as a quality-control tool for routine quantitative analysis of phenolic compounds in the different matrix. It opens up a number of application areas in health and environmental sciences, as well as measurements of body fluids in physiological and metabolic research.

Keywords: *Phenolic compounds, Folin Ciocalteu, on-line reaction, Ziziphus Jujuba.*

1. INTRODUCTION

Phenolic compounds are the biggest group of phytochemicals and many of them have been found in plant-based food. Polyphenols-rich diets have been linked to many health benefits. As excellent free radical scavengers, they offer high anti-oxidant capacity [1], so that, good protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases [2,3]. There has been much interest by scientist for integration of the experiments results in various disciplines, including biochemistry, cell biology, physiology, and pathophysiology, epidemiology, and food chemistry. It has been needed to identify and quantify the most effective phenolic compounds as biomarkers for dietary recommendations and for the formulation of new food products contributing to good health [4].

Since quantitative determination of phenolic compounds is hampered by their structural complexity and diversity, several methods have been reported for determining total phenolic compounds such as electrophoretic method [5], liquid chromatography [6,7,8,9], Flow injection Analysis [10,11], Fluor metric detection [12] and spectrophotometric detection [13]. Most of these methods are specific and require several standards. Colorimetric reactions are also widely used in the UV-Visible spectrophotometric detection, they are easy to perform and applicable in routine laboratory use [14]. Phenolic compounds react with Folin Ciocalteu

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as a specific redox reagent. They form a blue complex that can be quantified by visible-light spectrophotometry where the maximum absorption of the chromospheres depends on the alkaline solution and the concentration of phenolic compounds [15]. To obtain a complete reaction within short time, the use of an enormous excess of the reagent was needful as is reported by Folin et al. [16]. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. In the aim to solve this problem, to reduce time and reagent consuming, our purpose was to develop a simple automated method and to validate it, in accordance with International Conference on Harmonization (ICH) [17] guidelines, for quantitative analysis of total phenolic compounds.

The performance of the developed method was also checked by its application for *Ziziphus* Jujuba fruit investigation. In fact, this plant is a thorny Rhamnaceae tree, widely distributed in arid area with a great importance in preventing soil erosion and greening environment. Despite the abundance of this tree in Tunisia (Tunisian dorsal and Mejerda valley), no study has been reported. However, many studies have demonstrated that the extracts of Z. jujuba possesses diverse bioactivities and medicinal properties such as analgesic, ant diabetes and antifertility [18,19]. Rezla et al. [20] have also reported that the essential oil and the organic extracts of Z. jujuba seeds have antioxidant and antimicrobial activities against a diverse range of bacteria in vitro conditions, in addition of their potential for hair growth [21]. Therefore, it was great of interest to contribute to the valorization of this plant. The optimal extraction conditions for the maximum recovery of phenolic compounds were determined by applying the above developed and validated on-line method as a potential analytical tool.

2. MATERIAL AND METHODS

2.1. Solvants, reagents and solutions

All organic solvents and reagents used were of analytical grade. Double distilled water was used for the preparation of all solutions and as basis of the effluent. Folin Ciocalteu reagent was purchased from Merck, diluted in aqueous solutions with concentrations varied from 2,7% to 10,8 % (v/v). Sodium carbonate was from Sigma Aldrich, its concentration in the effluent was studied by varying it from 0,75 % to 1,5 (W/v).

To quantify total phenolic compounds, calibration curve with gallic acid (Sigma Aldrich) was used. For conventional method (off-line reaction and spectrophotometric detection), its concentration was set in the range of 400 to 3000 $mg.L^{-1}$ and in the range of 10 to 50 $mg.L^{-1}$ for the new developed method validation and the recovery extraction study.

2.2. Instruments

The on-line system includes an Ultimate 3000 Dionex micro pump, it allows the flowing carrier stream composed of Folin Ciocalteu reagent already mixed with Na_2CO_3 solution. The sample to be tested, is injected into the flowing carrier using a plugged WPS-3000 auto sampler system and will mix through the reaction coil (stainless tube with 2 m length and 0,01 mm i.d) streaming, before reaching the DAD 3000 Dionex UV/Visible detector.

2.3. On-line reaction procedure

The standard or sample solutions were injected (50 µL) into the carrier stream composed of Folin Ciocalteu solution (5,4% v/v), already mixed with Na_2CO_3 solution (0,75% W/v). Oxidation of the total phenolic compounds contained in the injected solution occures continuously through the reaction coil at 40°C and under a flow rate of 0,04 $mL.min^{-1}$. The blue colour product reached the DAD detector in 5 min and detected at 765 nm.

2.4. Validation of the developed method

Each experiment was run in triplicate and mean values were calculated.

The method was validated according to ICH guideline Q2 (R1) (2005) with respect to specificity, linearity, precision, and sensitivity.

The specificity was investigated by injecting different standard of phenolic compounds: gallic acid and tannic acid, separately and in a mixture. Sucrose as potential interference was also injected in the same operating conditions and all responses were compared to blank solution.

For the linearity study, three calibration curves at the range of 10 to 50 $\mu g.mL^{-1}$ were prepared from gallic acid standard solution. Linear fit was carried out using linear least square regression. Linearity response factors were calculated from the ratio between the peak area and gallic acid theoretical concentration.

Slope significance and proportionality tests were evaluated. Variance homogeneity and residues normality were checked with significance level of 0,05. Student's test was used to verify that the intercept (y at x = 0) is not significantly different from 0.

Precision was determined in five replicates of gallic acid standard solution (50 $\mu g.mL^{-1}$) on the same day (intra-day precision) and daily for 5 times over a period of three days (inter-day precision). The results were expressed as %RSD of the measurements.

The sensitivity was estimated by preparing a dilution series from the gallic acid 1$\mu g.mL^{-1}$ standard solution. Sufficient responses (peak area) were measured, based on signal to noise ratio evaluation at which : the analytic can be reliably detected ; limit of

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detection (LOD), typically three times the noise level and reproducibly quantified above the baseline noise, that gives S/N = 10 ; limit of quantitation (LOQ).

2.5. Plant material sample

Fresh Z. jujuba fruit was collected from the local area, in north west of Tunisia. The material was dried during 24 hours in hot air oven at 38°C, ground by a high speed blender and stored in air tight containers until further use.

2.6. Procedure for the extraction of total phenolic coumpounds from Z.jujuba

Z. jujuba prepared sample was investigated with solid-phase microwave assisted extraction procedure using a MILESTONE, START D microwave. The experimental conditions were chosen for the best phenolic compounds recovery. The accurate weighed plant powder of 500 mg were extracted with 10 mL of 60% aqueous ethanol, by means of 30 bar microwave pressure at 40°C temperature for 10 min. After the filtration through 0,2 µm membrane filter, the aliquot was tested for total phenolic compounds determination, by injecting (50 µL) into the on-line system and applying the developed method conditions.

3. RESULTS AND DISCUSSION

3.1. Effect of the flow rate on phenolic compounds oxidation by Folin Ciocalteu reagent

The effect of flow rate of carrier (composed of diluted Folin Ciocalteu reagent 2,7% (v/v) and sodium carbonate 0,75% (W/v) in double distilled water) at 40°C, was tested in the range of 0,03 to 0,4 mL/min, for all studied concentrations range of gallic acid (10 µg.mL^{-1} to 50 µg.mL^{-1}) injection. As it is shown in figure 1, the sensitivity slightly incresed with an increase of flow rate up to 0,04 mL/min. At faster flow rates, the signals decreased indicating that the reaction of phenolic compounds oxidation was partially or not occurred. Therefore, flow rate of 0,04 mL/min was chosen for further experiment.

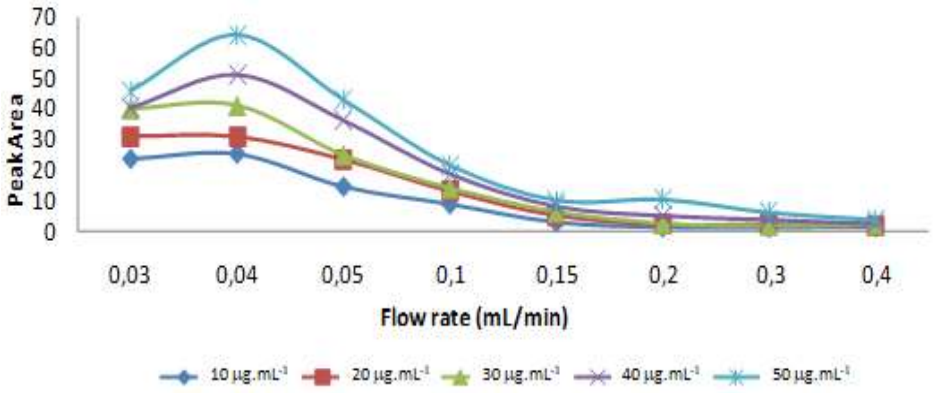

Figure 1. Study of the flow rate effect on the oxidation of gallic acid (10-50 µg.ml^{-1}) by Folin-Ciocalteu reagent in the on-line system.

3.2. Effect of the reaction coil temperature on phenolic compounds oxidation by Folin Ciocalteu reagent

Another way to accelerate oxidation of total phenolic compound with Folin Ciocalteu reagent was to control temperature effect during flowing into the reaction coil. The effect of the temperature was tested in the range from 40°C to 70°C. The results obtained showed in figure 2 that the absorbance decreased with the increase of temperature up to 50°C, and then it remained almost constant.

By using higher temperatures, undesirable reactions such as degradation and faster carrier stream occurred and resulted to loose in the oxidation recovery. Therefore, the temperature was fixed at 40°C and used for further study.

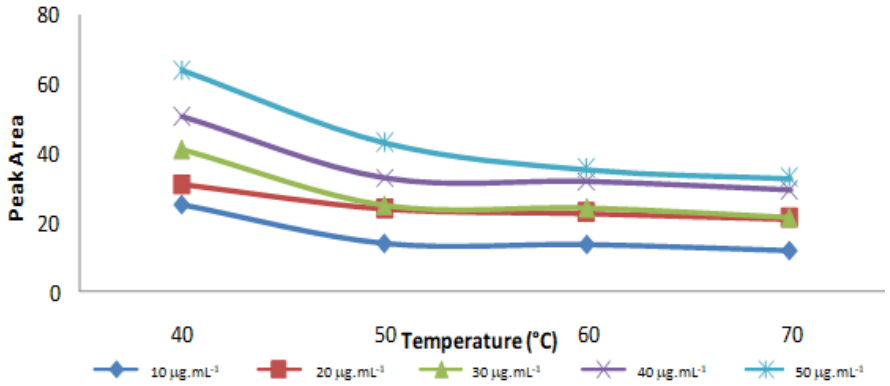

Figure 2. Study of the coil temperature effect on the oxidation of gallic acid (10-50 µg.ml^{-1}) by Folin Ciocalteu reagent in the on-line system.

3.3. Effect of Folin Ciocalteu concentration on phenolic compounds oxidation

The effect of the concentration of Folin Ciocalteu reagent in the carrier was examined at the following values : 2,7% ; 5,4% ; 8,1% and 10,8% (v/v). It can be seen in figure 3, that the signals became maximum at 5,4%.

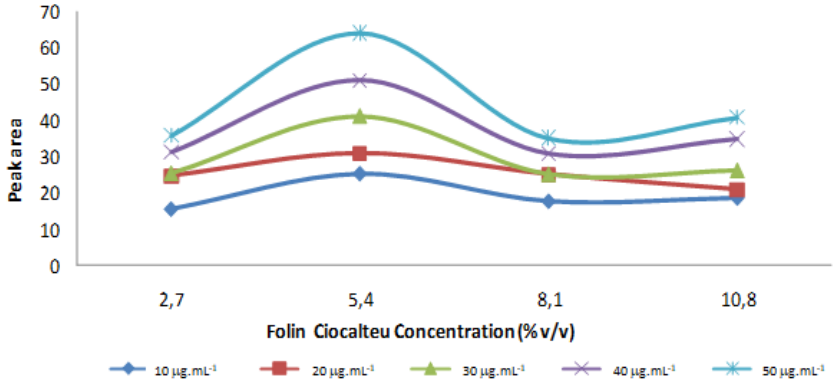

Figure 3. Study of the effect of Folin Ciocalteu concentration on the oxidation of gallic acid (10-50 µg.ml^{-1}) by in the on-line system.

The increase in the concentration of the reagent had an important effect in catalyzing the reaction and thus improving its kinetics, on the other hand this increase can lead to saturation of into the reaction coil and result to a non significant effect on the reaction recovery. In further experiments, 5,4 % Folin Ciocalteu concentration was selected.

3.4. Effect of sodium carbonate concentration on phenolic compounds oxidation by Folin Ciocalteu reagent

The Effect of the concentration of sodium carbonate was studied at 0,750%; 1,125% and 1,500% (W/v). No signficant effect on the oxidation recovery and the signals remained almost constant (figure4). The concentration of 0,750% (W/v) was selected for optimum on-line system.

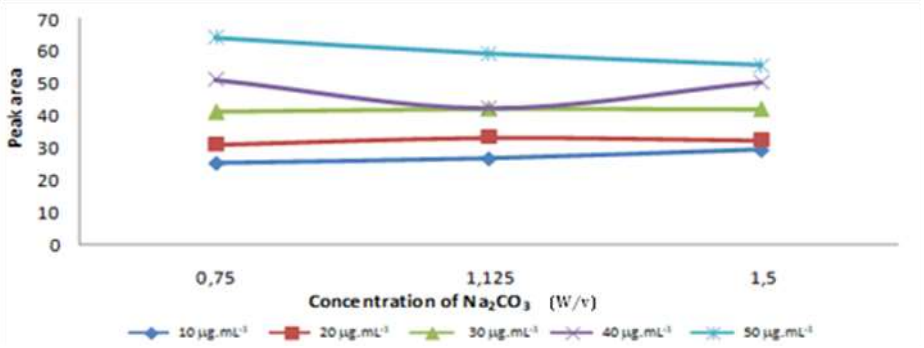

Figure 4. Study of sodium carbonate concentration effect on the oxidation of gallic acid (10-50 µg.ml^{-1}) by Folin Ciocalteu reagent in the on-line system.

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3.5. Method Validation

3.5.1. Specificity

The evaluation of the specificity of the method can be observed in Figures 5. It shows that all tested phenolic compounds had analysis signals in the same elution time and no significant interfering signals were observed when sucrose was injected in the operating conditions.

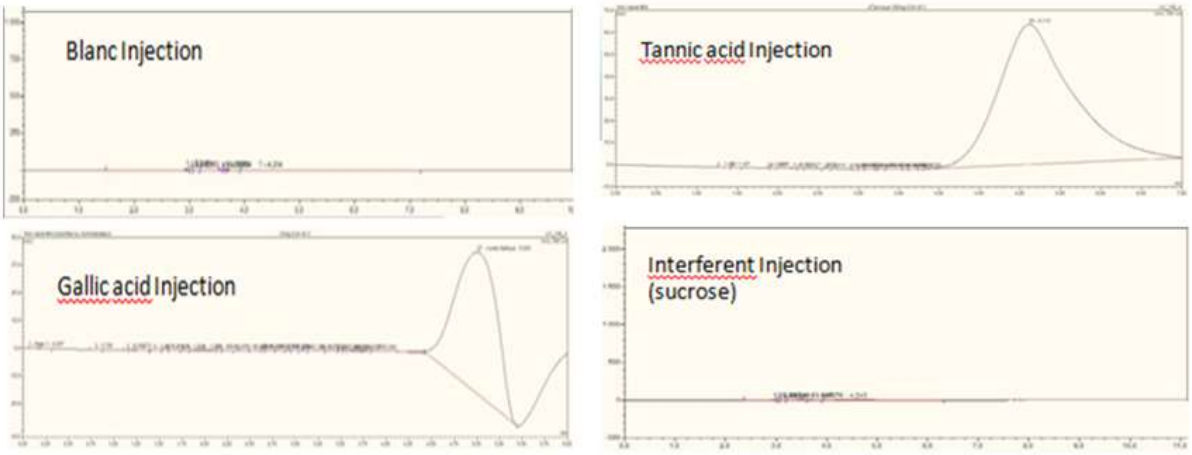

Figure 5. Signals for blank solution, gallic acid, tannic acid and sucrose (as interferent solution) for Specificity study.

3.5.2. Linearity

Resulting calibration curve follows the following equation: $y = 0{,}97\ x + 13{,}5$, where y is the peak area and x is the concentration of gallic acid (μg/mL). The correlation coefficient is close to the unit ($r = 0{,}992$), which shows a high probability of correlation between variables (area and concentration).

The variance analysis, confirms the homogeneity of variances ($C_{calculated} < C_{table}$) as shown in Table 1.

Table 1. Homogeneity of variances for the linearity study (Cochran's test)

Concentration level (i group)	**1**	**2**	**3**	**4**	**5**
Ni	3	3	3	3	3
variance of responses **Si^2**	0,0869	0,2923	0,2524	0,0441	0,1061
$C_{alculated}$	**0,3738**		**$C_{(0,05;5;2)\text{-table}}$**	**0,6838**	

In Table 2, the linearity significance of the curve and the normality of residues, which were randomly distributed without tendency, were demonstrated ($F_{calculated} > F_{table}$).

According to Student's test the intercept is not significantly different from zero, proving that the curve passes through the origin ($c_{alculated} = 89{,}2196 < t_{(0,05;13)\text{-table}} = 1{,}771$), fulfilling another analytic condition requirement.

Table2. Linearity significance for the linearity study (Fisher's test)

Variation due to	Degrees of Freedom	Variance	**$F_{calculated}$ S_I^2/S_R^2**	**$F_{(0,05;1;13)\text{-Table}}$**
Regression	1	S_I^2 =2862,501023		
Residuel	13	S_R^2 = 1,687901	**1695,89**	**4,67**

3.5.3. Precision

Precision was evaluated to identify variability due to random errors that cannot be controlled, as those related to the analysis time, reagents, instrument, and sample preparation [22]. For intra-and inter-day analysis, RSD ranged from 0,17% to à 0,34%. These values were in accordance with acceptable limits determined by the international requirements that establish a maximum RSD of 5% [23].

3.5.4. Sensitivity

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on sample containing very low concentrations of gallic acid (1µg.mL^{-1}). LOD was detected above baseline noise at S/N = 3. LOQ was reproducibly quantitated above the baseline noise at S/N = 10. In this study, LOD for a 50 µl injection of gallic acid standard was 0,01 ηg.mL^{-1}, and the LOQ was 0,03 ηg.mL^{-1}.

3.6. Application for the assay of total phenolic compounds in *Ziziphus* Jujuba fruit extract

The optimization of the adequate solvent polarity for the maximum extraction recovery of total phenolic compounds from *Ziziphus* Jujuba fruit raw material was carried out. Microwave assisted extraction was used at 40 °C temperature and 30 bar pressure, for 10min running time. The combination of 0,5g of the prepared solid raw material with 10 mL solvent extraction (aqueous ethanol) was the suitable solid-liquid ratio for total phenolic compounds extraction recovery testing.

The polarity of solvent extraction effect was performed by varying ethanol in water proportion from 0% to 100%. Table 3 illustrates the yield of total phenolic compounds obtained by the on-line new method ; resulted for each used extraction solvent.

Table3. Study of the effect of the solvent polarity on the extraction recovery of total phenolic compounds from *Ziziphus* Jujuba fruit by the on-line developed method

Extraction Solvent (ethanol :H_2O) (%)	0 :100	40 :60	50 :50	60 :40	100 :0
Total phenolic compounds Concentration (mg eqGA.g^{-1})*	63,07	65,40	87,06	66,60	27,40

*eqGA: equivalent of Gallic acid

4. CONCLUSION

The developed analytical system and method is suitable for its intended use. Its validation according to ICH and its application to *Ziziphus* Jujuba fruit investigation, generate both useful and meaningful data.

Its advantages in terms of specificity, linearity, precision, low LOD, low LOQ, time and reagents consuming provided its capability as quality-control tool for routine quantitative analysis of phenolic compounds in different matrix, and for investigating plant materials and biomass in health and environmental sciences, as well as measurements of body fluids in physiological and metabolic research are proved.

5. REFERENCES

[1] N.S Gaulejac, Y. Glories, N. Vivas, « Free radical scaving effect of anthocyanins in red wines », Food Res. Int., 1999 , vol. 32, pp. 327-333.

[2] B.A. Graf, P.E. Milbury, J.B. Blumberg, « Flavonols, flavonones, flavanones and human health: Epidemological evidence », J. Med. Food, 2005, vol. 8, pp. 281-290.

[3] I.C.W. Arts, P.C.H. Hollman, "Polyphenols and disease risk in epidemiologic studies", Am. J. Clin. Nutr., 2005, vol. 81, pp. 317-325.

[4] A. Scalbert, T.I. Johnson, M. Saltmarsh, "Polyphenols: antioxidants and beyond1–3", Am. J. Clin. Nutr., 2005, vol. 81(suppl), pp. 215S-7S.

[5] G. Cartoni, F. Coccioli, R. Jasionowska, "Cappillary electrophoretic separation of phenolic acids", J. Chromatogr. A., 1995, vol. 709, pp. 209-214.

DOI: 10.31695/IJASRE.2018.32711

[6] D.A. Guille, C.G. Barroso, J.A. Perez-Bustamante, "Selection of column and gradient for the separation of polyphenols in sherry wine by high performance liquid chromatographic method for the determination of phenolic compounds and furans in fortified wines", J. Chromatogr. A., 1996, vol. 724, pp. 117-124.

[7] P. Ho, T.A. Hogg, M.C.M. Silva, "Application of a liquid chromatographic method for the determination of phenolic compounds and furans in fortified wines", Food Chem., 1999, vol. 64, pp. 115-122.

[8] S. Tial, S. Nakamura, T.Cui, "High performance chromatographic determination of phenolic compound in rice", J. Chromatogr. A., 2005, vol. 1063, pp. 121-128.

[9] P. Vinas, C. Erroz, J. Hernandez, H. Cordoba, "Determination of phenols in wines by liquid chromatography with photodiode array fluorescence detection", J. Chromatogr. A., 2000, vol. 871, pp. 85-93.

[10] J.W. Schoonen, M.G.F. Sales, "Determination of polyphenols in wines by reaction with 4-aminoantipyrine and photometric flow-injection analysis", Anal. Bioanal. Chem., 2002, vol. 372, pp. 822-828.

[11] K. Leamsomrong, M. Suttajit, P. Chantiratikul, "Flow injection analysis system for the determination of total phenolic compounds by using Folin-Ciocalteu assay", Asian J App. Sciences, 2009, vol. 2 (2), pp. 184-190.

[12] S. Ossipova, V. Ossipov, E. Haukioja, J. Loponen, K. Pihlaja, "Proanthocyanidins of mountain birch leaves: Quantification and properties", Phytochem. Anal., 2001, Vol. 12, pp. 128-133.

[13] M.I.G. Pelozo, M.L.C. Cardoso, J.C.P. Mello, "Spectrophotometric determination of tannins and caffeine in preparations from *Paullinia. cupana* var. *sorbilis*", Braz. Arch. Biol. Technol., 2008, vol. 51, pp. 447-451.

[14] B.I. Giner-Chavez, « Condensed tannins in tropical forages ». Cornell University, Ithaca, NY, USA, 1996, (dissertation).

[15] European Pharmacopoeia, Council of Europe, "Determination of tannins in herbal drugs", Int. 6th ed. European Directorate for the Quality of Medicines, France : Strasbourg, 2007, A286.

[16] O. Folin, V. Ciocalteu, "On tyrosine and tryptophane determinations in proteins", J. Biol. Chem., 1927, vol. 73, pp. 627-650.

[17] ICH, Q2(R1), "Validation of analytical procedures: text and methodology in Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use", Switzerland : Geneva, 2005.

[18] S.P. Ambasta, "Useful plants of India Publications and Information Directorate" CSIR. India : New Delhi, 1986, pp. 703.

[19] A. Erenmemisoglu, F. Keletimur, A.H. Koker, H. Utsuol, Y. Tekol, M. Ustdal, "Hypoglycemic activity of Zizyphus jujube", Journal of Pharmacy and Pharmacology, 1995,vol. 47, pp. 72-74.

[20] M.S. Al-Reza, A. Rahman, J. Lee, S.C. Kang, "Potential roles of essential oil and organic extracts of *Zizyphus* jujuba in inhibiting food-borne pathogens", Food Chemistry, 2010, vol. 119, pp. 981-986.

[21] J.I. Yoon, M.S. Al-Reza, S.C. Kang, "Hair growth promoting effect of *Zizyphus* jujuba essential oil", Food and Chemical Toxicology, 2010, vol. 48, pp. 1350-1354.

[22] M.S.S. Cunha-Filho, C.A.T. Goncalves, P.R. SantosSoares, L.C. Sa-Barreto, R. Martinez-Pacheco, M. Landin, "Validationof analytical method and development of dissolution test for the antineoplastic beta-lapachone", Latin American Journal of Pharmacy, 2009, vol. 6, pp. 805-811.

[23] S. Zhang, K.S. Lovejoy, J.E. Shima, "Organic cation transporters are determinants of oxaliplatin cytotoxicity" Cancer Research, 2006, vol. 66(17), pp. 8847-8857.