Potential Activity of Fraction of Piper Betle Linn Against *Aggregatibacter Actinomycetemcomitans*

Rusyanti Yanti[1]*, Rusminah N[1], Hendiani I[1], Komara I[1]

1. Department of Periodontics Faculty of Dentistry, Universitas Padjajaran, Bandung, Indonesia.

Abstract

Aggregatibacter actinomycetemcomitans (Aa) is a potentially periodontal bacteria causing periodontal tissue destruction, which may not be cleaned at the time of scaling and root planing. Piper betle Linn which has long been used as a traditional medicine in Indonesia to cure various diseases in the mouth, potentially also as an adjunctive therapy in periodontitis. The objective of the research was to investigate the effect of betel leaf fraction on the growth of Aa bacteria. Materials and methods: Fresh betel leaf extraction with water, n-hexane, ethyl acetate and ethanol and 95% ethanol extract (EtOH) showed the highest inhibitory effect against Aa bacteria. 95% EtOH fractionated yielded 6 active fractions, then tested its antibacterial activity against Aa. Results: Fractionation of EtOH 95% yielded 6 active fractions comprising FrEtOH 30%, 50%, 100%, 10%, 20% and FW 100%. The test results of the 95% EtOH fraction showed 4 fractions that have inhibitory power against Aa bacteria. Conclusion: The 100% EtOH fraction shows the highest inhibitory effect against Aa bacteria.

Clinical article (J Int Dent Med Res 2018; 11(2): pp. 700-706)

Keywords: Fraction, Piper Betle Linn, Aggregatibacter, Actinomycetemcomitans.

Received date: 28 February 2018 **Accept date:** 06 April 2018

Introduction

Since 1930 the efficacy of betel leaf (Piper betle, Linn) has been known as traditional medicine in Indonesia that can cope with various diseases/abnormalities such as swelling, bad breath due to tooth decay, stop gum bleeding and clean the wound after tooth extraction. Pradhan conducted a literature review of betel leaf as an anti-inflammatory, diuretic, can improve blood circulation and help overcome bleeding. The results of research conducted by Suwondo and Hartono showed that gargling with betel leaf infusion can decrease plaque index and gingivitis. Dzen and Winarsih stated that the minimum concentration of betel leaf water 10% already have antimicrobial power.[1-4]

Periodontitis is an infectious disease caused by bacteria and causes damage to the periodontal ligament and alveolar bone to form a periodontal pocket. The most commonly associated periopathogenic bacteria with periodontitis are Aggregatibacter actinomycetemcomitans (Aa), Aa associated with initiation of periodontitis disease.[5] The mechanical action of scaling and root planing is the basis of treatment of periodontitis in order to purge the bacteria, calculus and cementum nectotics. The bacteria present at the bottom of the pocket may not be thoroughly cleaned by mechanical therapy, requiring chemical therapy to eliminate all residual bacteria left behind. Systemic antimicrobial administration may be one of the chemical treatment options but may have side effects such as allergic reactions, gastrointestinal disorders, and resistance to certain antimicrobials. In addition, the bioavailability of antibiotics has decreased when the drug is distributed to the periodontal tissue, so it is necessary to develop topical antimicrobial administration that can be administered directly to the infected area of the periodontal pocket. In addition, it is also necessary substances to maintain the antimicrobial influence remains high in the pocket so that antimicrobials can be absorbed by the network longer and released slowly.

Corresponding author:
Rusyanti Yanti
Department of Periodontics Faculty of Dentistry
Universitas Padjajaran
Bandung, Indonesia
E-mail: yanti.rusyanti@fkg.unpad.ac.id

The research of betel leaf extract showed an antibacterial effect on Aa bacteria. The results of phytochemical leaf gel betel leaf screening showed the presence of alkaloids, flavonoids, polyphenols, monoterpenoids, and sesquiterpenoids, which have antibacterial power potential characterized by the inhibition zone against Aa bacteria. Local gels of betel leaf extract (Piper betle L) as additional treatment of scaling and root planing in patients with chronic periodontitis can decrease pocket depth and acquire epithelial attachment level.[6]

Until now there has been no single study using betel leaf fraction as an antibacterial agent in bacteria that cause periodontal disease, especially periodontitis disease. Therefore, the researcher is interested to examine the antimicrobial effect on betel leaf fractions to Aa growth as the main etiology bacteria of periodontitis disease.

Materials and methods

Collection, Water Content, and Phytochemical Samples Analysis

Betel leaf plants were taken from the garden Conservation and Aquaculture Unit Biofarmaka, Tropical Biofarmaka Study Center LPPM IPB located in the Campus IPB Dramaga Bogor. Betel leaves are harvested and selected from the no-shoots. Once collected, the sample is washed to clean the remains of soil or dirt that are still attached (Figure 1). Since the wet sample is very susceptible to microbial growth, it is necessary to dry the process. Drying aims to get samples that can be stored for a longer time. The water content of the sample should be below 10%. The drying method used is solar radiation for 3-4 days.

Figure 1. Betel leaves are still fresh.

Figure 2. Betel leaf that has been dried.

And finally stored using a plastic bag that was given a silica gel dryer. The dried samples were then determined by their gravimetric method (Figure 2). In addition, also conducted qualitative analysis of phytochemicals to betel leaf powder.

Solvent extraction

Dry betel leaves are pollinated before being extracted with various solvents. The solvents used were water, ethanol 50%, 95% ethanol, and hexane. A total of 50g of samples were macerated in a solvent with a ratio of 1:10. The mixture was incubated for one day, then separated by filtration. The filtrate is dried by a rotary evaporator (Figure 3).

Figure 3. Extract of betel leaf from 4 solvents.

Antibacterial Test

The AA antibacterial test was performed by a paper disc diffusion method. Suspension of test bacteria that has been prepared smeared evenly on the agar medium. Inside is then placed paper discs that have been spilled with solvent as a negative control, chlorhexidine solution as a positive control and sample extract solution with various concentrations. The concentration series

of extracts used were 1.25%, 2.5%, 5%, 10%, 20% and 40%. Media for subsequent incubation in an anaerobic box. Clear areas around the paper disc are measured as obstacles.

Fractionation Extract

The selected active extract is further fractionated by using column chromatography (Figure 4). The column used is HP 20 so that the chromatographic method chosen is the reversed phase. The fractionation was done by gradient elution so that the ethanol fraction was 10%, 20%, 30%, 50% and 100%, respectively (Figure 5).

Figure 4. Chromatographic process.

Figure 5. Fractionation of column chromatography from ethanol extract 95%.

Statistical analysis

All experiments were carried out in triplicate. Data points were represented by mean of the measured values. Statistical analysis was carried out using MS-Excel software

Results and Discussions

Phytochemical Powder and Rendement of Betel Leaf Extract

Before extraction, dry powder of betel leaf is first determined the water content and phytochemical identity. The extracted betel leaves have a moisture content of 2.33%. The recommended level for a simplicial, which is recommended as a moisture content below 10%. Qualitative identification of the chemical content of betel leaf powder shows the presence of phenol (flavonoid), tannin and terpenoid compounds. this result is in accordance with the test conducted by Arawwawala which identifies the existence of three groups of chemical compounds on betel leaves.[7]

Extraction was performed using four different types of solvents with different polarity ranges. Water, ethanol 50%, 95% ethanol, and hexane are solvents in polar to nonpolar sequences. The use of these four types of solvents is expected to produce extracts that can represent various types of chemical compounds present in betel leaves. The four solvents produce a dark yellowish-green extract with a slightly liquid and dry consistency. The result of extraction of betel leaf by maceration resulted in the yield of extract in the range of 2 - 18% (Table 1). Water extract is an extract with the largest rendement and hexane extract as the smallest yield. These data indicate that the extractive polar component of betel leaf is greater than the non-polar component.

Extract Type	Rendement (%)
Water Extract	17.7
EtOH extract 50%	11.6
EtOH extract 95%	6.3
Hexane extract	2.4

Table 1. Rendemen data of betel leaf extract from various solvents.

Antibacterial Activity Leaf Betel Extract

To demonstrate the presence of compounds in betel leaf that may play a role in the prevention or treatment of periodontal disease, trials have been conducted to exclude the growth of AA bacteria commonly used as a target in oral disease.[8] The inhibitory ability of extract on Aa bacteria growth is seen from the clear zone around the placement of the paper disc with the extract depleted as shown in Figure 6.

Of the four tested extracts, 95% ethanol extract showed the highest relative inhibitory activity in the concentration range of 1.25% - 40% (Table 2 and Table 3) to Aa bacteria. At 40% extract concentration, ethanol 95% extract

gave relative resistance about 40% compared to positive control (chlorhexidine) while Water extract showed no obstacle in Aa bacteria.

The results showed that ethanol extract from betel leaves showed the inhibitory activity against Aa bacteria. The existence of this activity may be due to the content of phenolic compounds of alilpirokatekol contained in the extract.[9,10,11]

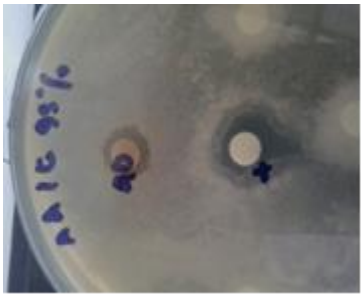

Figure 6. Zone of 95% ethanol extract barrier to Aa

Extract	Weight Extract (g)	Rendement (%)	Zone of Inhibition (mm) in Concentration (%)									
			1.25	2.5	5	K+	K-	10	20	40	K+	K-
			Bacteria AA									
Water	8.896	17.71	6	6	6	11.25	6	6	6	6	12.42	6
Ethanol 50%	5.8743	11.59	6	6	6	11.08	6	6.11	6.85	6.37	11.52	6
Ethanol 95%	3.1837	6.28	6	6	6	11.36	6	6	7.52	8.83	11.49	6
Hexane	1.226	2.42	6	6	6	10.75	6	6	6	6.2	12.82	6

Table 2. Relative resistance zone of betel leaf extract in various concentrations against Aa. K + = Chlorhexidine, K- = Negative Control

Extract Type / Concentration	% Relative resistance Extracts against Aa*					
	1.25%	2.50%	5%	10%	20%	40%
Water Extract	0	0	0	0	0	0
Etoh Extract 50%	0	0	0	1.22	9.982	4.18
Etoh Extract 95%	0	0	0	0	18.78	38.27
Hexane Extract	0	0	0	0	0	2.17
Positive Control (KP)	100					
Negative Controls (KN)	0					

Table 3. Percent of relative obstacles of betel leaf extract in various concentrations against Aa bacteria. *% Obstacles = (area of extract area of KN area) / (area of KP-area of KN) x 100%

Fraction and Nature of its bioactivity

Of the four solvents attempted for the extraction of the betel leaf chemical component ie water, ethanol 50%, 95% Ethanol and hexane. Ethanol 95% (EtOH95%) yielded extracts which showed the highest inhibition against Aa bacteria. Further elucidation to determine the active component responsible for the activity was done by fractionating EtOH95% extract using column chromatography method. The reversed phase chromatography system is utilized in this fractionation using the HP-20 diaion silent phase. The separation is done gradually with the ethanol mixture phase, water with ethanol composition from 0-100%. Fractionation with this gradient system yielded 6 fraction types with yield as shown in Table 4. The fraction yield ranged from 0.4 to 31.9% range. The highest yield was produced by FrEtOH10% fraction of 31.9% while the lowest yield was obtained by FrEtOH30% fraction of 0.4%.

Inhibition tests against AA bacteria were used to obtain the most active fraction of 6 available fractions. The fraction activity test results are listed in Table 5. The fraction and extract inhibitory tests were performed at a concentration of 10% (10000 ppm). At this concentration, EtOH95% extract did not reveal inhibitory power for AA bacteria. The existence of the fractionation process, causing the separation of the active component and causing the occurrence of the

active compound concentration increases its inhibitory properties against AA bacteria at the concentrations tested. Of the six fractions obtained, 2 fractions have no inhibitory power while the other 4 have inhibitory activity. The highest inhibitory power is given by FrEtOH100% fraction. The EtOH10% fraction, although yielding the highest yield, the chemical component therein does not exhibit barrier activity. This shows down the part of the EtOH95% extract that likes water (the more polar component in the extract) is inactive. In general, fractions that have inhibitory activity also exhibit antioxidant activity.

Fraction	Rendement (%)*
FrEtOH30%	0.4
FrEtOH50%	26.5
FrEtOH100%	15.6
FrEtOH10%	31.9
FrEtOH20%	11.3
FW100%	6.2

Table 4. Rendemen fraction of fractionation result from EtOH95% extract of betel leaf. *This fractionation rendemen error of ± 8%.

Fraction Type / Extract Activity	% Obstacles Aa
FrEtOH30%	17.5
FrEtOH50%	30.5
FrEtOH100%	48.5
FrEtOH10%	0.0
FrEtOH20%	0.0
FW100%	26.5
Etoh Extract 95%	0.0
Positive Control (KP)	100
Negative Controls (KN)	0

Table 5. Inhibition of betel leaf fraction to Aa bacteria. *% Obstacles = (area of extract area of KN area)/(area of KP-area of KN) x 100% # Cannot be determined IC50 in the range of concentrations tested

Thin Layer Chromatography (TLC) Analysis Ethanol extract 95%

To determine the chemical characteristics of the fraction, Thin Layer Chromatography (TLC) analysis was performed on fraction and 95% ethanol extract. Ethanol extracts 95% and six fractions were applied to the Silica gel 60 F254 TLC plate, then inserted into a chromatographic vessel that has been saturated with a solution of the developer (Toluene: ethyl acetate (93: 7).The plate is eluted to the extent of development. dried and observed under UV light and calculated Rf value.

Fractionation EtOH Extract 95%

95% EtOH leaf extract weighed 0.8 gram and dissolved in 20 ml of 30% ethanol. Chromatography columns prepared with HP 20 stationary phase. The soluble extract was incorporated into the column and eluted with 80 ml of 30% ethanol. Eluat chromatographic results are accommodated in Erlenmeyer and hereafter referred to as Fraction 1 (F1 / FrEtOH30%). The column was re-eluted with 100 mL of 50% ethanol and the eluate was collected (Fraction 2 / F2 / FrEtOH50%) and continued with 100 mL 100% ethanol (Fraction 3 / F3 / FrEtOH100%). A chromatography column was welded with 100% ethanol (FW100%). Fraction 1 was then added to 200 ml of distilled water and re-inserted into the column for eluting and eluat accommodated in Erlenmeyer (Fraction 4 / F4 / FrEtOH10%). The columns were re-eluted with 100 ml of 20% ethanol (Fraction 5 / F5 / Fr 20 O 20%), 100 mL of ethanol 30% (Fraction1 / F1 / FrEtOH30%) and terminated by a washing process with 100 mL 100% ethanol (FW100%). The entire fraction then tested its inhibitory activity against AA bacteria.

Determination of KHM and KBM by dilution method for EtOH's Most Active Faction 95%

Against the most active faction, KHM and KBM values were determined. Prepared Eppendorf sterile was then characterized from concentrations of 100%, 50%, 25%, 12.5%, 6.25%, control (-), control (+) chlorhexidine. Eppendorf is filled with BHIB medium with 0.5 ml volume at concentrations of 50%, 25%, 12.5%, 6.25%. Fr3 (10000 ppm) samples were inserted (100%) as 1 mL in Eppendorf marked 100%. For 0.5 ml dilution in 100% Eppendorf was taken, then put in 50% Eppendorf to 1 ml and the dilution was 1/2 = 50%. Furthermore, the dilution dilated by taking and entering 0.5ml from 50% Eppendorf into 25% Eppendorf so that the

screening is 1/4 = 25%. Next, take 0.5ml of Eppendorf 25% then put on Eppendorf 12.5% so the filter is 1/8 = 12.5%. Last take 0.5ml of Eppendorf 12.5%, then put on Eppendorf 6.25%, so the filtering becomes 1/16 = 6.25%. Then the rest is removed 0.5ml from Eppendorf 6.25% so the whole Eppendorf contains 0.5ml. After the series dilution is completed, into Eppendorf is included as much as 0.5ml of bacteria that has been equated with turbidity Mc.Farland 0.5 (1.5 x 103 CFU / ml). Incubation was performed in the incubator for 24 hours at 37 ° C anaerobically. Then 0.1ml is taken from each Eppendorf and is grown on Muller Hinton media. Inoculum is grown with technical spreading then incubated at 37 ° C anaerobically 24 hours. Observations were made on the growth of bacteria and counting colonies growing on Muller Hinton media.

Chemical Profile Faction and Ekstark Leaf Betel

To show how the chemical composition of the results of the fractionation process has been done, TLC analysis of fractions and extracts. Figure 7 shows the TLC separation results using silica gel plates and eluent Toluene: ethyl acetate (93: 7). Most of the active chemical components of the extract are concentrated in F2 and F3. F1, F5 and FW100% contain polar components that prefer the stationary phase versus its mobile phase. All three provide a 1 spot result but provide different barrier properties. F5 is not active. Other fractions provide a good separation even though there are still some components that are more restrained by the stationary phase. F2 and F3 show a similar separation and both have inhibitory activity. Since F4 has no resistance, it can be assumed that the chemical components responsible for the inhibitory activity of F2 and F3 are chemical compounds at Rf values 0.32, 0.6, and 0.07.

The research data indicate that the most active F3 fraction inhibits the growth of Aa bacteria and the F2 & F6 fraction inhibits bacterial growth with almost same resembling zone values, whereas fraction F1 has the smallest inhibitory zone value. Two other fractions, F4 & F5 are inactive against the Aa bacteria. When compared to the standard/control compound zone values, F3 is closest to the control value.

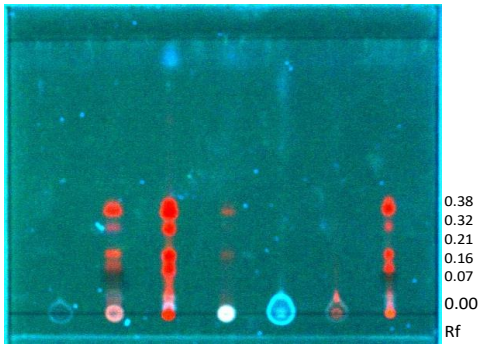

Figure 7. Results of TLC analysis of the fraction and extract under UV 366nm.

ES Fraction Concentration (%)	Number of Colonies (Cfu) Aa		
	1	2	3
100	0.00	0.00	0.00
50	0.00	0.00	0.00
25	0.00	0.00	0.00
12.5	10	9	13
6.25	43	39	34
Positive Control CH	0.00	0.00	0.00
Negative Controls	162	155	171

Table 6. Ethanol fraction test results of 95% betel leaf against Aa bacteria.

Test Sample	Zone of Inhibition (mm)		
	1	2	3
ES	0.00	0.00	0.00
F1	13.00	12.80	12.70
F2	16.60	17.00	17.20
F3	21.20	21.00	22.00
F4	0.00	0.00	0.00
F5	0.00	0.00	0.00
F6	16.00	15.80	15.60
Positive Control CH	30.20	30.60	31.20
Negative Controls	0.00	0.00	0.00

Table 7. Results of inhibitory test Fraction Ethanol Extract 95% Betel Leaves Against Aa Bacteria.

Several other studies suggest that LED exposure at a distance of 2 cm with a density of 20.48 J / cm2 quantum yield decreases the amount of A. actinomycetemcomitans bacteria by 81%. This result is mainly due to the use of

Chlorophyll from Alfalfa Medicago sativa L as exogenous photosensitizer, which can utilize 77% of light with a wavelength of 453 nm. This research method uses phenol to obtain and characterize and purify LPS A. actinomycetemcomitans. Furthermore, it was performed on experimental animals (Wistar rats). This is done by calculating the levels of IgA, sIgA, and IgG by ELISA technique. This study produces LPS A. actinomycetemcomitans in accordance with E. coli O127 LPS which is the LPS standard. In animal experiments, the results showed that serum IgA levels had increased although there was no significant difference with the control group from the statistical test, whereas the level of IgA in saliva showed a significant difference when compared with the control group and the treatment group. The level of IgG in the treatment group also appears to be increasing although statistical analysis did not show significant differences from the controlled group.[12, 13]

Conclusions

This study shows that 95% betel leaf ethanol extract has the potential to inhibit the growth of bacteria that cause periodontal disease such as Aa bacteria. The most active chemical component of betel leaf is polar soluble in ethanol which is shown from the result of 100% EtOH leaf fraction which gives the highest resistance compared to the other four fractions.

Further activities that need to be done include chemical identification of the most active fraction either by authentic standards or through isolation steps and molecular structure determination. Furthermore, the development of AA antibacterial material formulation can be done with the active ingredients contained in betel leaves.

References

1. Pradhan D, Suri K.A, Pradhan D.K, Biswaroy K. Golden Heart of Nature: Piper Betle L. Journal of Pharmacognosy and Phytochemistry 2013;1(6):147-167.
2. Suwondo S. The Study of Medicinal Plants Used By The Indonesian People in The Treatment of dental and oral diseases with the aim of improving the efficiency Of Its Use. Dissertation of Padjadjaran University 1993.
3. Hartono S.W. The Influence of Boiled Water Betel Leaf As A Mouthwash Against The Gums. FKG UNPAD 1993.
4. Dzen S.M, Roekistiningsih S.S, Winarsih S. Bacteriology of Medical, Bayumedia Publishing, Malang 2003;16-18,133-140.
5. Amaliya, A, Laine M.L, Delanghe J.R, Loos B.G, Van Wijk A.J, Van der Velden U. Java Project on Periodontal Diseases: Periodontal Bone Loss In Relation To Environmental And Systemic Conditions. J Clin Periodontol 2015; 42:325–332. doi: 10.1111/jcpe.12381.
6. Rusminah, N. Wendari, S, Susanto, A. Long-Term Evaluation of the Effectiveness and Safety of Local Leaf Gel Gelih Gelih in Periodontitis Patients. Final Report of Research Unemployment of Higher Education UNPAD 2013.
7. Arawwawala, L.D.A.M, Hewageegana, H.G.S.P, Arambewela, L.S.R, Ariyawansa, H.S. Standardization of Spray-Dried Powder of Piper Betle Hot Water Extract. Pharmacognosy Magazine 2011;7(26):157-160.
8. Palombo, E.A. Traditional Medicinal Plant Extracts And Natural Products With Activity Against Oral Bacteria: Potential Application In The Prevention And Treatment of Oral Diseases. Evidence-based Complementary and Alternative Medicine 2011; art. no. 680354
9. Punareewattana, K, Aiemsaard, J. Primary Screening of antibacterial activity of Piper Betle Leaf Extract Against Porphyromonas Gingivalis, The Bacterium Causing Periodontitis. KKU Veterinary Journal 2016;26(2):101-113.
10. Teanpaisan, R, Kawsud, P, Pahumunto, N, Puripattanavong, J. Screening For Antibacterial And Antibiofilm Activity In Thai Medicinal Plant Extracts Against Oral Microorganisms. Journal of Traditional and Complementary Medicine 2017;7(2):172–177.
11. Ramji, N, Ramji, N, Iyer, R, Chandrasekaran, S. Phenolic Antibacterial From Piper Betle In The Prevention of Halitosis. J. Ethnopharmacology 2002;83:149-152.
12. Ernie, M.S, Suryani, D.A, A. H. Zaidan. An in vitro Anti-microbial Photodynamic Therapy (aPDT) with Blue LEDs to Activate Chlorophylls of Alfalfa Medicago sativa L on Aggregatibacter actinomycetemcomitans. J Int Dent Med Res. 2016; 9(2):118-125.
13. Rini, D.R, Sidarningsih, Tuti, K. Immuogenic Potency of LPS Actinobacillus Actinomycetemcomitans Local Isolate on Iga, Siga, and Igg Titre in Aggressive Periodontitis. J Int Dent Med Res 2017;10(1):128-133.
14. Bera D, Lahiri D, Nag A. Novel natural antioxidant for stabilization of edible oil: The ajowan (Carumcopticum) extract case. J Am Oil ChemSoc 2004;81(2):169–72.
15. Currie E, Schulze A, Zechner R, Walther TC, Farese RV. Cellular fatty acid metabolism and cancer. Cell Metab 2013;18(2):153–61.
16. Butler M, Huzel N, Barnabe N, Gray T, Bajno L. Linoleic acid improves the robustness of cells in agitated cultures. Cytotechnology 1999;30(1–3):27–36.
17. Hisha H, Kohdera U, Hirayama M, et al. Treatment of Shwachman Syndrome by Japanese Herbal Medicine (Juzen-Taiho-To): Stimulatory effects of its fatty acids on hemopoiesis in patients. Stem Cells 2002;20(4):311–19.
18. Mainou-Fowler T, Proctor SJ, Dickinson AM. Gamma-linolenic acid induces apoptosis in B-chronic lymphocytic leukaemia cells in vitro. Leuk Lymphoma 2001; 40(3-4):393–403.
19. Lima TM, Kanunfre CC, Pompéia C, Verlengia R, Curi R. Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis. Toxicol in vitro 2002;16(6):741–7.
20. Vaisey-Genser M, Morris DH. Introduction History of the cultivation and uses of flaxseed. In: Muir AD, Westcott ND, eds. Flax: The genus Linum. London: Taylor & Francis;2003:1–21.
21. Marlen S, Sibylle A, Andr S, Barbara N, Birgit P, Volker B, Dagmar-Ulrike R. Effects of extracts from Linumusitatissimum on cell vitality, proliferation and cytotoxicity in human breast cancer cell lines. J Med Plants Res 2014;8(5):237–45.
22. Hausott B, Greger H, Marian B. Naturally occurring lignans efficiently induce apoptosis in colorectal tumor cells. J cancer Res ClinOncol 2003;129(10): 569-576.
23. Waszkowiak, K., Gliszczyńska-Świgło, A, Barthet V, Skręty J. Effect of extraction method on the phenolic and cyanogenicglucoside profile of flaxseed extracts and their antioxidant capacity. J Am Oil Chem Soc. 2015; 92(11–12): 1609–19.
24. Waszkowiak K, Gliszczyńska-Świgło A. Binary ethanol–water solvents affect phenolic profile and antioxidant capacity of flaxseed extracts. Eur Food Res Technol 2016;242(5): 777-86.