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Optimization of Flavonoid Extraction from the peel of Poovan variety Banana (*Musa acuminata*) using L_{16} Orthogonal Design

A. Annu[1*], R. Baskar[2], C. Sivasankari[3]
[1]Department of Industrial Biotechnology, Government College of Technology, Coimbatore -13, India.
[2]Department of Biotechnology, Kumaraguru College of Technology, Coimbatore-49, India.
[3]Department of Chemistry, Government College of Technology, Coimbatore -13, India.
***Corresponding Author: E Mail address: annualagarsamy@gct.ac.in**

ABSTRACT

The main aim of the work is to explore the effect of single factors such as temperature, extraction time, solvent concentration and material ratio on the extraction of flavonoid contents present in the peel of Poovan variety banana i.e *Musa acuminata.* Water, ethyl acetate, ethanol and methanol were the solvents chosen for the extraction process. L_{16} Orthogonal designs of experiments were conducted to identify the optimal conditions for the effective extraction of flavonoids. With aqueous condition, the optimal amounts of flavonoids were obtained at 95˚C for 1 hour with 1:20 material ratio. Similarly with ethyl acetate as solvent, the flavonoid extraction reached its maxima at 65˚C, for 2 hours and a material ratio of 1:20. When ethanol was used as solvent, the optimal extraction conditions were found at 60˚C, 4 hours incubation time, 95% solvent with 1:20 material ratio. Whereas with methanol as an extracting agent, the maximum yield of flavonoids were identified at 65˚C, 3 hours incubation time, 75% solvent with 1:20 material ratio. Comparing all the above four solvents, ethanol gave the optimal yield of flavonoids followed by ethyl acetate, water and methanol respectively. One way ANOVA was performed to study the influence of each factor on optimum flavonoid extraction and it was observed that material ratio was the significant factor that affected the extraction procedure.

KEY WORDS: ANOVA, Flavonoids, L_{16} Orthogonal design, *Musa acuminata*, Solvents.

1. INTRODUCTION

From an environmental perspective, it is inevitable that the by-products of the plants produced by the agro-based industries be reused. Fruit peels generally harbor a fairly large quantity of non-nutritional antioxidants, including flavonoids, flavones, and polyphenol (Ciou, 2008). After citrus, banana is the second largest produced fruit, contributing about 16% of the world's total fruit production **(**Debabandya Mohapatra, 2010). Banana peel accounts for 40% of the total weight of fresh bananas or plantains and these peels are currently used as either fertilizer or discarded without any purpose in many countries **(**Eun-Hye Lee, 2010). Banana and Plantain peels are major agricultural wastes which have been used as medicine, animal feeds, blacking of leathers, soap making, fillers in rubber and so on (Arawande and Komolafe, 2010). According to the criteria established by the National Cancer Standard Institute **(**Eun-Hye Lee, 2010)**,** banana peel extract is classified as non-toxic to human cells and hence it can be safely utilized as a natural source of antioxidants.

Flavonoids are a broad range of secondary metabolites that occur ubiquitously in foods of plant origin. They belong to the group of polyphenolic compounds that exhibit strong antioxidant property and thus protecting the cells against damaging effects of reactive oxygen species (Om and Kim, 2008). They also have long been recognized to possess anti-inflammatory, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities (Tapas, 2008). Hence there is a need for effective use of natural antioxidants which have low side effects (Nagavani and Rao, 2010) and due to their minimal side effects, there are growing interests in using natural products for preventive and therapeutic medicine (Jo, 2008).

Previous studies on the antioxidant potential of the peel extracts of nine different local banana varieties (*Musa sapientum*) reported that the content of flavonoids was higher in Poovan banana and was highly correlated with its lipid per oxidation inhibition activity **(**Ramakrishnan Baskar, 2011)**.** Hence the present study has been focused on the optimization of flavonoid extraction from the poovan variety banana peel for maximum yield. A good experimental design is highly needed to optimize all the factors which greatly influence the extraction process. Orthogonal design experiments **(**Sampath, 2013) were adopted in this study which focus on the main effects of the factors and thus reduce the number of experiments drastically.

2. MATERIALS AND METHODS

2.1. Plant material: Banana (*Musa acuminata*) fruits were purchased from local market without any ethylene treatment and were identified and authenticated for their scientific names by Dr.T.N.Balamohan, Professor & Head, Department of Fruits & Crops, Horticulture College & Research Institute, TNAU, Coimbatore. The peels were separated and cut into pieces. It was then shade dried and ground into coarse powder.

2.2. Extraction process: The main factors which greatly influence the extraction process such as temperature, extraction time, material ratio (weight of sample: volume of extracting agent) and extracting agent (%) were studied individually. The L_{16} (4^4 & 4^3) Orthogonal design of experiments i.e four levels and four different parameters were carried out to determine the optimum extraction conditions. To investigate the effect of each parameter on extraction process, a single factor analysis of variance (One way ANOVA) was adopted.

2.3. Estimation of Total Flavonoid Content (TFC): TFC was estimated spectrophotometrically (Thiyagarajan Sathiskumar, 2012) with slight modification. About 0.5 ml of the extracted sample was mixed with 1.25ml of distilled water. To this 0.075ml of 5% sodium nitrite was added and it was allowed to stand for 5 minutes. To the sample, 0.15ml of 10% aluminium chloride was added followed by the addition of 0.5 ml of 1 M sodium hydroxide was added and the absorbance was measured at 510 nm. Rutin was used as a standard for the construction of calibration curve. Data was reported as mean ± SD for three replicate measurements.

3. RESULTS AND DISCUSSION

Previous reports for the extraction optimization of total flavonoids from microwave-pretreated *Shatiln pomelo* fruit peel revealed that a solid:liquid ratio of 1:8 for 40 min at 70°C with 80% ethanol was required for best extraction efficiency of total flavonoids **(**ZHU Yuan-Ping, 2009). In this experiment the fruit peel slices of *Shatiln pomelo* was microwave pretreated for 3 min at 495 W before extraction. A similar research has been done on the dried *Citrus grandis* tomentosa in which narigin was extracted using an orthogonal experiment (L_9 $(3)^4$) for optimum extraction condition and the effect of each extraction factor through a single-factor exploration. The results depicted that the extraction yield decreased in the following order: extraction time> ultrasonic power> extraction temperature> ratio of solvent to raw material. The highest yield was obtained when temperature, ratio of solvent to raw material, ultrasonic time and ultrasonic power were 40°C, 20:1, 50 min, and 280 W, respectively **(**Fansheng Kong et al., 2013)**.**

A previous study has been carried out on the contents of flavonoids present in the leaves of *Tabernaemontana heyneana Wall* which depicted that the amount of flavonoids extracted reached its maxima when extracted at 85°C for 2 hrs by using 75% ethanol with a material ratio of 1:05 and 4 times of extraction **(**Sathishkumar, 2008). It is also reported that temperature was found to be a significant factor that affects the extraction procedure. A similar work was carried out on *Musa acuminata* peel in which the total flavonoid contents were extracted with methanol using rotary vaccum evaporator (Manmohan Singh, 2013). Also the hexane extract was prepared using Soxhlet apparatus after defatting with petroleum ether which was then concentrated under vacuum to get solid crude mass**.** In general, to carry out a complete evaluation of the effect of four different parameters at four different levels on the yield of flavonoids would require (4^4) 256 experiments. But the total numbers of experiments were reduced to 16 using L_{16} Orthogonal design graph.

3.1. Effect of Temperature on flavonoid extraction: Fig1.a showed that the contents of flavonoids tend to increase gradually with a rise in the temperature ranging from 65 to 95˚C. At higher temperature, the cell wall gets ruptured and the flavonoids and other cell components can easily diffuse out into the extracting agent**.** Hence the optimum temperature for aqueous extract was found to be 95˚C.

Fig 1.b showed that the flavonoid content increased with increase in temperature from 45 to 65˚C, but after which it decreases. This may be due to the fact that the flavonoids could be oxidized at temperature of surpassing 65°C, so that the contents of flavonoids extracted started to decrease gradually. Fig 1.d indicated that the contents of flavonoids increased with a rise in the temperature in a range of 35ºC to 45ºC with a 10ºC temperature interval. Followed by a small drop in 55˚C, the contents of flavonoids increased to the maximum at 65˚C. It may be probable that the greater speed of the molecule movements occur at higher temperature, so that flavonoids diffused more quickly from cell to extracting agent (Sathishkumar, 2008). Hence the optimized temperature for the extraction of flavonoids using ethyl acetate and that of methanol as solvent was 65˚C.

Fig 1.c depicted that the contents of flavonoids tend to increase gradually with a rise in the temperature ranging from 50ºC to 60ºC for ethanol after which it decreases. Effect of temperature on extraction is dual. On one hand, higher temperature can accelerate the solvent flow and thus increase the flavonoids content and on the other hand, higher temperature can decrease the fluid density that may reduce the extraction efficiency **(**Thiyagarajan Sathiskumar, 2012)**.** Hence, it was found that 60ºC was the optimum temperature for extracting the flavonoids using ethanol as solvent.

3.2. Effect of extraction time on flavonoid extraction: The result of Fig 2 showed that the optimized time for extraction was 1 hr for aqueous extract after which the flavonoid content gets decreased and on the other hand for ethyl acetate extract it was 2 hrs. The contents of flavonoids extracted, increased gradually with the increase in extraction time for ethanolic extract. The contents of flavonoids extracted for 4 hrs reached its maxima. Whereas for methanolic extract, the flavonoid content reached its maxima at 3 hrs of extraction time after which it reduced. There is a kind of fluctuation in the flavonoid content extracted and these results may be due to the synergistic

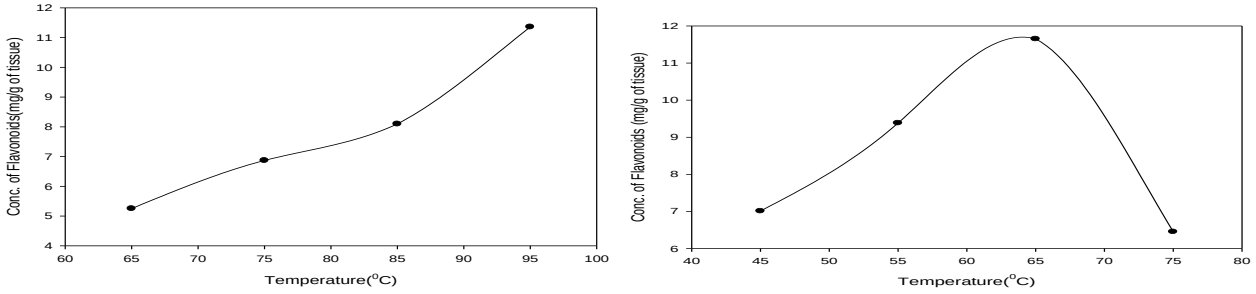
effect of other parameters involved. Increase in time may lead to an increase in adhesion of diffused particle around the walls of supporting material like glass or test tubes that may hinder the extraction process (Felicetti, 2008).

3.3. Effect of extracting agent on flavonoid extraction: The result of Fig 3 revealed that the contents of flavonoids increased with the concentration of ethanol i.e., 65% to 95%. The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In this study, the results indicated that the optimal ethanol concentration for extraction of flavonoids was found to be 95%. In case of methanol, the contents of flavonoids increased with the increase in concentration i.e., 65% to 75%. A decrease in the flavonoids content was noticed furthermore, i.e., beyond 75%. Usually the addition of a small amount of liquid modifier can enhance the efficiency of extraction effectively, thus the extraction time is reduced and there is an improvement in the recovery of different types of natural products from plants (Liu, 2008).

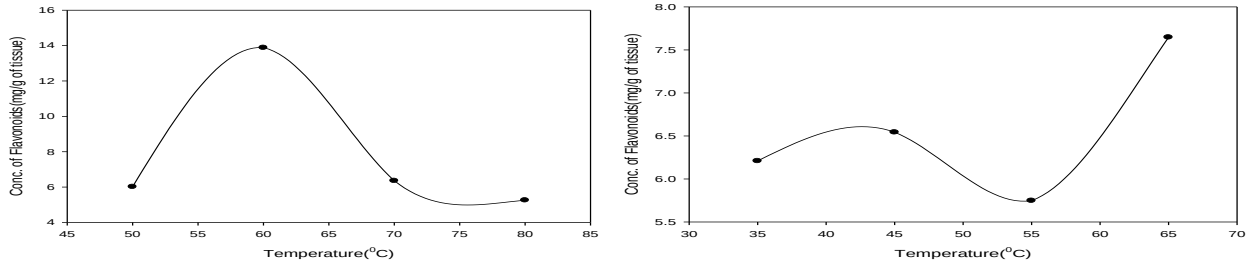
3.4. Effect of material ratio on flavonoid extraction: Fig 4 showed the contents of flavonoids extracted were maxima at 1:20 materials ratio. A significant rise in the flavonoids content was observed with the material ratio of 1:20 for all the four solvents. A gradual increase in the flavonoids content was noticed when there is an increase in the material ratio. Generally when the extraction volume is increased it can cause swelling of the material and hence the absorption of the constituent. A similar report (Xiao, 2008) has showed that higher solvent volume may give lower yield which is totally inversed when compared with conventional extraction technique. In contrast, another study (Chen, 2009) suggested that when more volume of solvent is used for a fixed amount of raw material, the more dilute effect occurs in solvent side. This gave a larger concentration difference between interior of plant cells and external solvent thus resulting in faster extraction rate. The basic mechanism is that the increase in the ratio of solvent to raw material could decrease solution concentration difference to the inside and outside of plant cells which consequently accelerated the diffusion of solute particles and made more flavonoids to reach the solution.

3.5. Optimization of flavonoids extraction using L_{16} orthogonal design: The parameters and orthogonal design of experiments for the extraction of flavonoids were given in table 1 and 2 respectively. The results were shown in the form of range of analysis and one way ANOVA by DMRT software in table 3, 4, 5 and 6. For both aqueous and ethyl acetate extracts, the order of effects of each factors on flavonoid extraction were C>A>B. Whereas for ethanolic and methanolic extract, the order of factors were D>A>B>C and D>C>A>B respectively. In all the four solvents, material ratio had the greatest effect on extraction and it was significantly different at 5% level. An equivalent effect was observed with temperature change in ethanolic extract, but it was not significantly different at 5% level. The other factors did not play much vital role in other solvents for optimum extraction of flavonoids. From statistical analysis, the optimum extraction conditions were $A_2B_3C_1$, $A_2B_3C_1$, $A_2B_3C_4D_1$, $A_3B_4C_2D_1$ for water, ethyl acetate, ethanol and methanol respectively. It means that with aqueous condition, the optimal amounts of flavonoids were obtained at 95˚C for 1 hour with 1:20 material ratio. Similarly with ethyl acetate as solvent, the flavonoid extraction reached its maxima at 65˚C, for 2 hours and a material ratio of 1:20. When ethanol was used as solvent, the optimal extraction conditions were found at 60˚C, 4 hours incubation time, 95% solvent with 1:20 material ratio. Whereas with methanol as an extracting agent, 65˚C, 3 hours incubation time, 75% solvent and 1:20 material ratio were the optimum conditions for flavonoid recovery.

3.6. Comparision of yield of Flavonoids: Fig 5 showed that the yield of flavonoids was higher in ethanolic extract than other three extracts. However, the yield of flavonoids from the aqueous and ethyl acetate was also very near to that of the ethanolic extracts. The concentration of flavonoids in aqueous extract was 11.36(mg/g tissue), ethanolic extract was 13.884(mg/g tissue), methanolic extract was 7.647(mg/g tissue) and ethyl acetatae was 9.384(mg/g tissue). The order of maximum yield of flavonoids from four different solvents were ethanol> water> ethyl acetate> methanol. Hence it was concluded that the best solvent used for the optimum extraction of flavonoids from banana peel was ethanol. Ethanol could be a right choice since it is environmentally benign, relatively safe to human health and interacts with flavonoids probably through non covalent interaction and hence promote a rapid diffusion to the solution (Thiyagarajan Sathiskumar, 2012). Next to ethanol, water may be chosen as a suitable solvent for flavonoid extraction mainly when the flavonoid applications are concentrated on food related industries.



Effect of Temperature on flavonoid extraction using Fig 1.a Water as solvent, Fig 1.b Ethyl acetate as solvent



Effect of Temperature on flavonoid extraction using Fig 1.c Ethanol as solvent, Fig 1.d Methanol as solvent

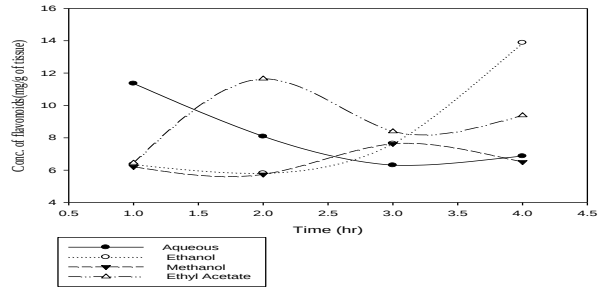


Fig.2.Effect of extraction time on flavonoids

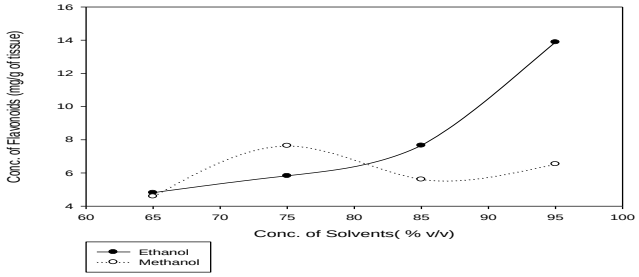


Fig.3.Effect of extracting agent on flavonoid extraction

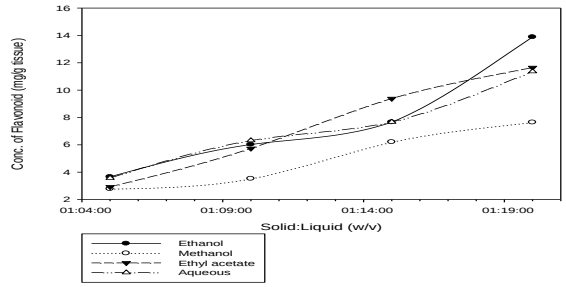


Fig.4.Effect of material ratio on flavonoid extraction

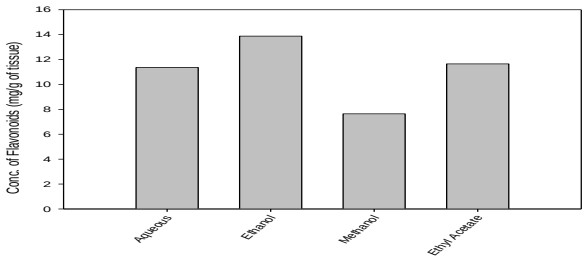


Fig.5.Comparision of yield of flavonoids from aqueous, ethanol, methanol and ethyl acetate extracts

Table.1.Factors for the extraction of flavonoids

Levels	Temperature(˚C) (A)				Ext time (hr) (B)	Solvent (%) (C)	Sol:liq (w/v) (D)
	Water	Ethyl acetate	Ethanol	Methanol			
1.	65	45	50	35	1	65	1:05
2.	75	55	60	45	2	75	1:10
3.	85	65	70	55	3	85	1:15
4.	95	75	80	65	4	95	1:20

(Solvent (%) i.e factor C does not correspond to Water and Ethyl acetate)

Table.2.L_{16} Orthogonal design (4^4 level)

Exp.	A	B	C	D
1	1	1	2	3
2	1	2	1	4
3	1	3	4	1
4	1	4	3	2
6	2	2	2	2
7	2	3	3	3
8	2	4	4	4
9	3	1	3	4
10	3	2	4	3
11	3	3	1	2
12	3	4	2	1
13	4	1	4	2
14	4	2	3	1
15	4	3	2	4
16	4	4	1	3

(For water and ethyl acetate L_{16} Orthogonal design (4^3 level) is adopted in which the factor D is omitted in Table 2)

Table.3.Yield of flavonoids from aqueous and ethyl acetate extracts

EXP		A	B	C	Flavonoids (mg/g)	
					Aqueous	Ethyl acetate
1.		1	1	1	$1.94^{a} \pm 0.005$	$2.52^{b} \pm 0.01$
2.		1	2	2	$3.32^{c} \pm 0.007$	$4.64^{f} \pm 0.02$
3.		1	3	3	$4.94^{f} \pm 0.015$	$5.89^{i} \pm 0.03$
4.		1	4	4	$5.25^{f} \pm 0.016$	$7.01^{l} \pm 0.04$
5.		2	1	2	$5.31^{g} \pm 0.004$	$5.73^{h} \pm 0.01$
6.		2	2	1	$3.14^{h} \pm 0.002$	$2.05^{a} \pm 0.004$
7.		2	3	4	$5.45^{b} \pm 0.020$	$8.39^{m} \pm 0.02$
8.		2	4	3	$6.87^{i} \pm 0.009$	$9.38^{n} \pm 0.01$
9.		3	1	3	$6.35^{m} \pm 0.009$	$5.26^{g} \pm 0.02$
10.		**3**	**2**	**4**	**$8.09^{0} \pm 0.019$**	**$11.65^{o} \pm 0.02$**
11.		3	3	1	$3.49^{d} \pm 0.004$	$2.05^{a} \pm 0.004$
12.		3	4	2	$6.00^{j} \pm 0.008$	$4.23^{d} \pm 0.01$
13.		**4**	**1**	**4**	**$11.36^{p} \pm 0.016$**	$6.45^{k} \pm 0.02$
14.		4	2	3	$7.67^{n} \pm 0.017$	$5.94^{j} \pm 0.01$
15.		4	3	2	$6.31^{k} \pm 0.008$	$4.54^{e} \pm 0.006$
16.		4	4	1	$3.57^{e} \pm 0.003$	$2.92^{c} \pm 0.003$
K_1	Aqueous	3.87	6.24	3.04		
	Ethyl acaetate	5.02	4.99	2.39		
K_2	Aqueous	5.20	5.56	5.24		
	Ethyl acaetate	6.39	6.08	4.79		
K_3	Aqueous	5.99	5.08	6.46		
	Ethyl acaetate	5.80	5.22	6.62		
K_4	Aqueous	7.23	5.43	7.54		
	Ethyl acaetate	4.97	5.89	7.82		
k_1	Aqueous	0.97	1.56	0.76		
	Ethyl acaetate	1.26	1.25	0.60		
k_2	Aqueous	1.30	1.39	1.31		
	Ethyl acaetate	1.60	1.52	1.20		
k_3	Aqueous	1.50	1.27	1.62		
	Ethyl acaetate	1.60	1.52	1.20		
k_4	Aqueous	1.81	1.36	1.89		
	Ethyl acaetate	1.24	1.47	1.96		
R	Aqueous	0.84	0.29	1.13		
	Ethyl acaetate	0.36	0.27	1.36		

Values represent mean ± SD of 3 replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT.

Table.4.ONE WAY ANOVA for aqueous and ethyl acetate extracts

Levels	Sum of squares		Degrees of freedom	Mean square		F-values	
	Aqueous	Ethyl acetate		Aqueous	Ethyl acetate	Aqueous	Ethyl acetate
A	23.84	5.57	3	7.95	1.86	1.29	0.16
B	1.97	3.23	3	0.66	1.076	0.08	0.09
C	43.78	78.82	3	14.59	26.27	3.69	8.28
			9				

Table.5.ONE WAY ANOVA for Ethanolic and Methanolic extracts

Levels	Sum of squares		Degrees of freedom	Mean square		F-values	
	Ethanol	Methanol		Ethanol	Methanol	Ethanol	Methanol
A	36.17	0.86	3	12.06	0.29	1.93	0.07
B	22.54	0.70	3	7.51	0.23	1.02	0.06
C	11.72	3.18	3	3.91	1.06	0.47	0.29
D	36.37	37.23	3	12.12	0.79	1.95	4.95
			12				

Table.6.Yield of flavonoids from Ethanolic and Methanolic extracts

EXP		A	B	C	D	Flavonoids (mg/g)	
						Ethanol	Methanol
1.		1	1	1	3	4.55^{f} ± 0.04	6.21^{g} ± 0.34
2.		1	2	2	4	4.07^{e} ± 0.23	4.49^{g} ± 0.21
3.		1	3	3	1	3.49^{bc} ± 0.03	1.53^{a} ± 0.01
4.		1	4	4	2	6.02^{i} ± 0.30	3.52^{d} ± 0.02
5.		2	1	2	1	3.43^{bc} ± 0.01	2.22^{b} ± 0.03
6.		2	2	1	2	5.82^{i} ± 0.09	3.33^{d} ± 0.10
7.		2	3	4	3	7.65^{k} ± 0.08	5.61^{f} ± 0.16
8.		**2**	**4**	**3**	**4**	**13.88^{l} ± 0.34**	6.54^{h} ± 0.05
9.		3	1	3	4	6.36^{j} ± 0.09	4.54^{e} ± 0.10
10.		3	2	4	3	3.86^{de} ± 0.15	5.74^{f} ± 0.07
11.		3	3	1	2	3.29^{b} ± 0.02	3.26^{d} ± 0.08
12.		3	4	2	1	3.65^{cd} ± 0.03	2.38^{b} ± 0.13
13.		4	1	4	2	3.55^{c} ± 0.02	2.75^{c} ± 0.05
14.		4	2	3	1	2.18^{a} ± 0.01	2.77^{c} ± 0.17
15.		**4**	**3**	**2**	**4**	5.25^{h} ± 0.08	**7.64^{i} ± 0.40**
16.		4	4	1	3	4.81^{g} ± 0.04	4.59^{e} ± 0.03
K_1	Ethanol	4.53	4.47	3.90	3.19		
	Methanol	3.94	3.96	3.19	2.23		
K_2	Ethanol	7.70	3.99	4.82	4.67		
	Methanol	4.43	4.09	4.89	3.22		
K_3	Ethanol	4.29	4.93	5.56	5.22		
	Methanol	4.01	4.52	4.15	5.54		
K_4	Ethanol	3.95	7.09	6.20	7.39		
	Methanol	4.44	4.26	4.15	5.83		
k_1	Ethanol	1.13	1.12	0.97	0.80		
	Methanol	0.99	0.99	0.80	0.56		
k_2	Ethanol	1.93	0.10	1.21	1.17		
	Methanol	1.11	1.02	1.22	0.81		
k_3	Ethanol	1.07	1.23	1.39	1.31		
	Methanol	1.00	1.13	1.04	1.39		
k_4	Ethanol	0.99	1.77	1.55	1.85		
	Methanol	1.11	1.07	1.04	1.46		
R	Ethanol	0.94	0.77	0.58	1.05		
	Methanol	0.13	0.14	0.43	0.90		

Values represent mean ± SD of 3 replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT.

4. CONCLUSION

In conclusion, it is suggested that the optimized conditions for flavonoid extraction were found to be 95˚C, 1 hour and 1:20 material ratio for water; 65˚C, 2 hours and a material ratio of 1:20 for ethyl acetate; 60˚C, 4 hours incubation time, 95% solvent with 1:20 material ratio for ethanol and 65˚C, 3 hours incubation time, 75% solvent with 1:20 material ratio for methanol. Moreover, material ratio was found to be the significant factor that affects the extraction process. Among the four solvents, ethanol proved to be the best solvent for extraction yielding maximum flavonoid content. More research on flavonoid's biological activity has to be done in future research.

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