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# Optimal process for the extraction and identification of flavonoids from the leaves of *Polyalthia longifolia* using $L_{16}$ Orthogonal design of experiment

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## ABSTRACT

The study has been carried out to investigate the effects of single factors such as temperature, extraction time, concentration of ethanol, material ratio and no. of extractions on the contents of flavonoids present in the leaves of *Polyalthia longifolia.* On this basis, an $L_{16}$ orthogonal design of experiment was used to determine the optimal conditions for the extraction of flavonoids. The amount of flavonoids extracted reached its maxima at 65 ºC for 2 hrs by using 75% ethanol (modifier) with a material ratio of 1:10 and 2 times of extraction. The TLC performed for the optimal extracts showed the presence of rutin and quercetin related compounds.


**Keywords:** Annonaceae, Flavonoids, Orthogonal experiments, Single-factor experiments, *Polyalthia longifolia.*

## INTRODUCTION

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities that make them medicinally important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, flavonoids, glycosides, tannins, and volatile oils, possess medicinal properties.

Flavonoids are the most important and prominent phenolic compounds that are known for their antioxidant and free radical scavenging activity (Van Acker et al., 1996). They have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They possess anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity (Cushnie and Lamb, 2005). Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancer and cardiovascular diseases.

In many cases, it is highly difficult to find quickly the suitable experimental conditions for a given separation task. Prediction of separation conditions is not yet straightforward. Therefore, good experimental design becomes increasingly important. Orthogonal array can be defined as a matrix with the columns representing the number of parameters to be studied with their different levels in different combinations of experiments and the number of rows equal to the number of experiments (Siva Hemalatha et al., 2007). Orthogonal design which only

focuses on the main effects of the factors, allows the number of experiments to be drastically reduced. In separation science, this kind of experimental design has already shown its usefulness in liquid chromatography and capillary electophoresis (Hu Zhide et al., 2002).

*Polyalthia longifolia* (Sonn.) Thwaites (Family: Annonaceae) is a tall handsome evergreen tree and cultivated all over India. Ethanobotanically, *Polyalthia longifolia* is used as anti-cancer, anti-inflammatory, analgesic, hepatoprotective, antiulcer and antihyperglycaemic agents (Malairajan et al., 2006). It is also used to treat anti-helmintic and kidney diseases. The *Polyalthia* genus has been investigated phytochemically and was reported to contain alkaloids, flavonoids, acetogenin and triterpenoids (Padmaa Paarakh and Khosa, 2009). Chromatographic analysis of the methanol extract of *Polyalthia longifolia* also revealed the presence of steroids, alkaloids, biterpenoids, carbohydrates, amino acids, essential oil, phenolics and flavonoids as major phytochemicals (Anupam Ghosh et al., 2008). Previous reports are there for the presence of several cytotoxic compounds like halimane diterpene, 3β,5β,16α-trihydroxyhalima-13(14)-en-15,16-olide and a new oxoprotoberberine alkaloid, (−)-8-oxopolyalthiaine from *Polyalthia longifolia* var. *pendula* (Yang-Chang Wu et al., 2000). Similarly, the ethanolic leaf extract of *Polyalthia longifolia* possess a potent nitric oxide radical scavenging activity (Moni Rani Saha et al., 2008).

In spite of several scientific documentation, so for no work has been progressed in investigating the optimization of flavonoids extraction from the leaves of this plant. So our laboratory has focused in determining the optimal conditions for the extraction using $L_{16}$ orthogonal design of experiment.

## MATERIALS AND METHODS

### Plant material

The plant leaves were collected from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India. The species was identified, confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India. The voucher specimen (No. SAM 01) was deposited at Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, Tamilnadu, India.

### Extraction process

The main factors that affect the extraction of flavonoids like temperature, extraction time, materials ratio, extracting agent (%) and the no. of extractions were studied separately. The optimum extraction conditions were then determined by $L_{16}$ orthogonal design of experiment (Table 1). A single factor analysis of variance (One way ANOVA) was espoused to investigate the effect of each factor in the extraction of flavonoids.

### Estimation of total flavonoids content (TFC)

Total flavonoids content (TFC) was estimated spectrophotometrically (Beckman DU 530 UV/Vis spectrophotometer, USA) with slight modifications (Zhishen et al., 1999). About 0.1 ml of the leaf extract added 4.9 ml of distilled water and 0.3 ml 5% $NaNO_2$. 3 ml of 10% $AlCl_3$ was added 5 minutes later. After 6 minutes, 2 ml of 1 M NaOH was added and the absorbance was measured at 510 nm. Rutin was used as a standard for constructing a calibration curve. Data were reported as mean ± SD for three replicate measurements.

### Identification of flavonoids by thin layer chromatography (TLC)

Chromatographies of the optimized extracts were run one dimensionally in the mobile phase solvent (ethyl acetate - water - ethanol, 5:5:1, v/ v/ v) at room temperature of 25 ºC. The concentrated extracts (50 µl) were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 5 mm. Authentic markers of flavonol (quercetin) and flavonoid glycoside (rutin) obtained commercially were co-chromatographed. Identification of the flavonoids in the extracts was done under far UV light after the application of ammonia as spraying agent (Adam et al., 2002; Guorong et al., 2006).

## RESULTS

The optimal conditions for the extraction of flavonoids was found to be at 65

ºC, 2 hrs extraction duration, 75% ethanol, 1:10 material ratio and 2 times of extraction (Table 2). The investigation proved that the temperature was found to be a major factor that affects the extraction procedure of flavonoids. Moreover, the TLC results of the optimized extracts revealed the presence of quercetin and rutin related compounds (Figure 5 and Figure 6).

## DISCUSSION

Flavonoids are a large family of polyphenolic compounds synthesized by plants (Beecher, 2003). Scientists are interested in the potential health benefits of flavonoids especially as nutraceuticals associated with fruits and vegetable-rich diets. Many of the biological effects of flavonoids appear to be related to their ability to modulate cell-signaling pathways (Williams et al., 2004), rather than their antioxidant activity. Higher intakes of flavonoid rich foods have been associated with reduced risk of various chronic diseases (Hirvonen et al., 2001), coronary heart disease (Knekt et al., 2002), cardiovascular disease (Yochum et al., 1999) and neurodegenerative diseases (Ramassamy, 2006). Various reports exist about the flavonoids action on the inhibition of the development of chemically-induced cancers in animal models of lung ( Yang et al., 1998), oral (Balasubramanian and Govindasamy, 1996), esophageal (Li et al., 2002), stomach (Yamane et al., 1996), colon (Guo et al., 2004), skin (Huang et al., 1997), prostate (Gupta et al., 2001; Haddad et al., 2006) and breast cancer (Yamagishi et al., 2002). Previous reports for the extraction optimization of flavonoids from the leaves of *Tabernaemontana heyneana* Wall has revealed that a Solid: liquid ratio of 1:05 for 2 hours at 85 ºC was required for higher yield (Sathishkumar et al., 2008).

### Effect of temperature on the extraction of flavonoids

Figure 1 showed the contents of raw flavonoids increasing gradually with a rise in the temperature from 55 ºC to 85 ºC. The contents of flavonoids gradually increased with an interval of 10 ºC. It may be plausible that the greater speed of the molecule movements may be facilitated at higher temperature so that the flavonoids diffused more quickly from cell to extracting agent. But the flavonoids could be oxidized if the temperature exceeds 80 °C and as a result the contents of flavonoids extracted will start to decrease gradually (Yaqin et al., 2005). Temperature's effect 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, it can decrease the fluid density that may reduce the extraction efficiency (He Guo-qing et al., 2005). The overall analysis revealed that 65 ºC was the optimum temperature for the extraction of raw flavonoids.

### Effect of flavonoids extraction time

The result of Figure 2 showed that the contents of flavonoids extracted for 2 hrs reached maxima and prolonged extraction may not yield an increased content. Furthermore a decrease in the flavonoids content was noticed for 3 hrs extraction and a sudden increase in their content was observed for 4 hrs extraction time. This increase in the flavonoids content may be due to the synergistic effect of other parameters involved.

### Effect of material ratio on the extraction of flavonoids

Figure 3 showed at 1:10 material ratio the contents of raw flavonoids extracted reached the maxima. Further increase in the material ratio leads to a gradual decrease in the flavonoids content resulting in a saturated condition. This decrease might be due to the fact that when the material ratio reached a certain level, the extract was well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase (Yaqin et al., 2005).

### Effect of extracting agent (ethanol) on the extraction of flavonoids

The result of Figure 4 revealed that the contents of raw flavonoids extract increases with the concentration of ethanol i.e., 65% and 75%. On further increase in the ethanol concentration, i.e., beyond 75% leads to a decrease in the flavonoids content. Among various solvents, ethanol was selected as a right choice because it is environmentally benevolent and comparatively safe to human health (He Guo-qing et al., 2005). Generally,

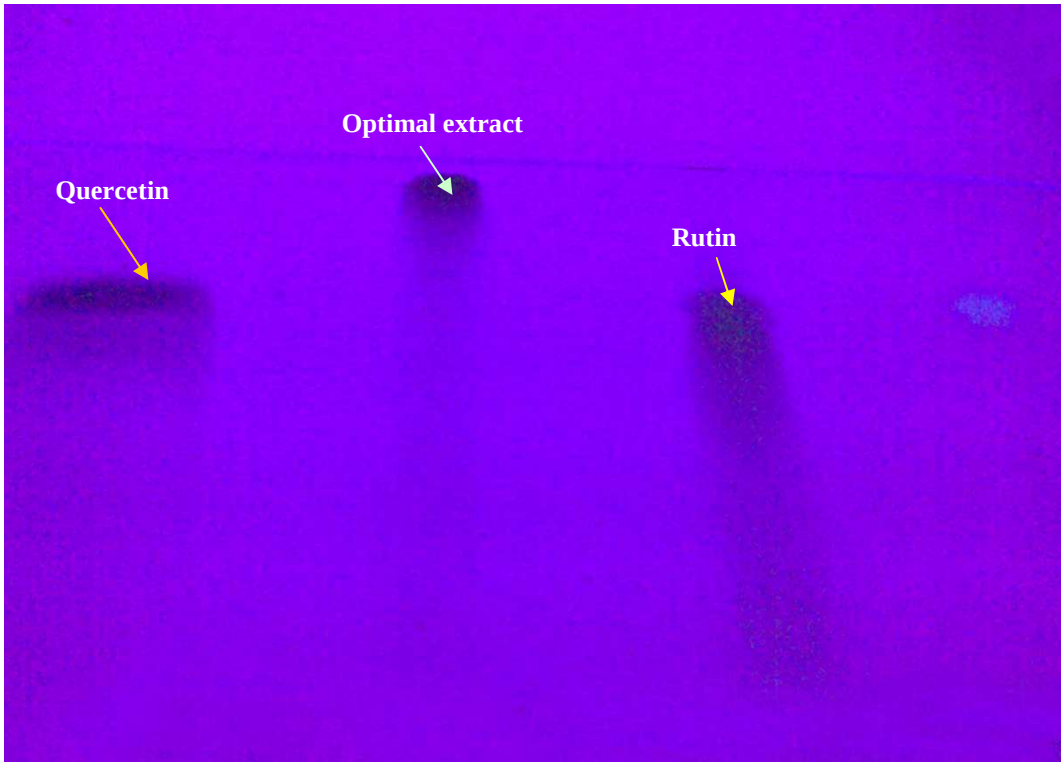


**Figure 5:** Identification of flavonoids from optimised extract by TLC under Far UV light.

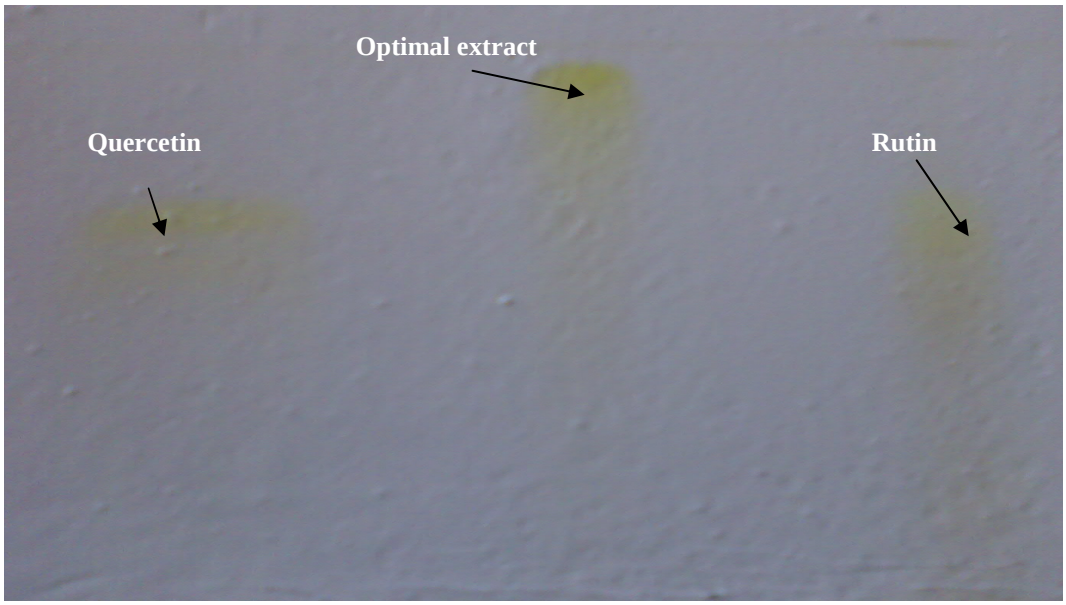


**Figure 6:** Identification of rutin related compounds by TLC under visible light (Enlarged image using Iflex image vision).

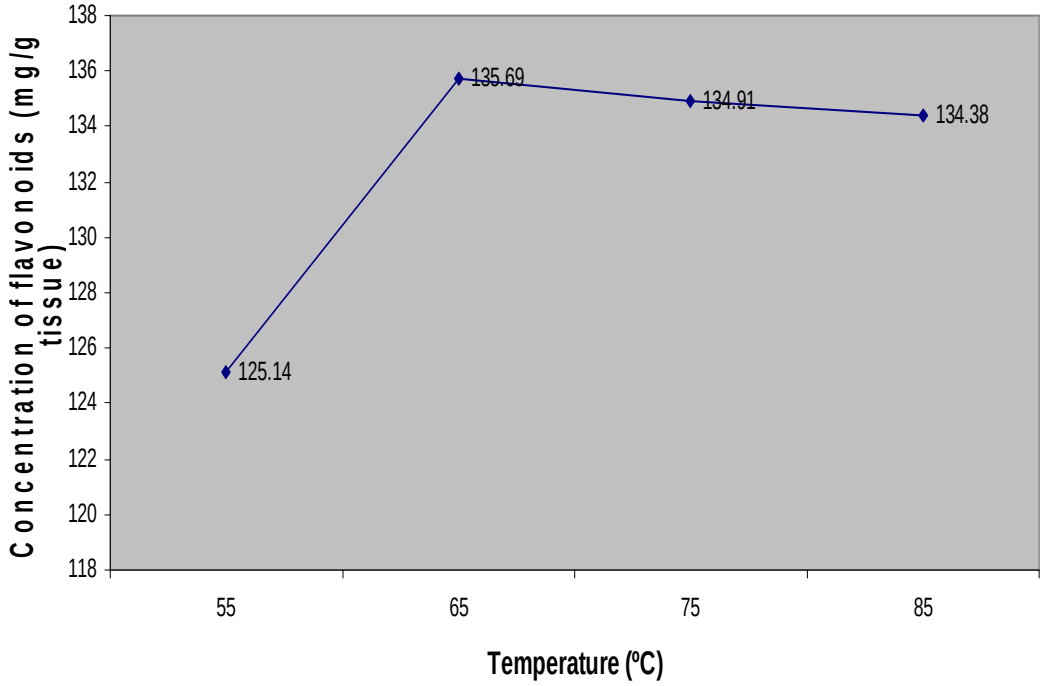


**Figure 1:** Effect of temperature on flavonoids extraction.

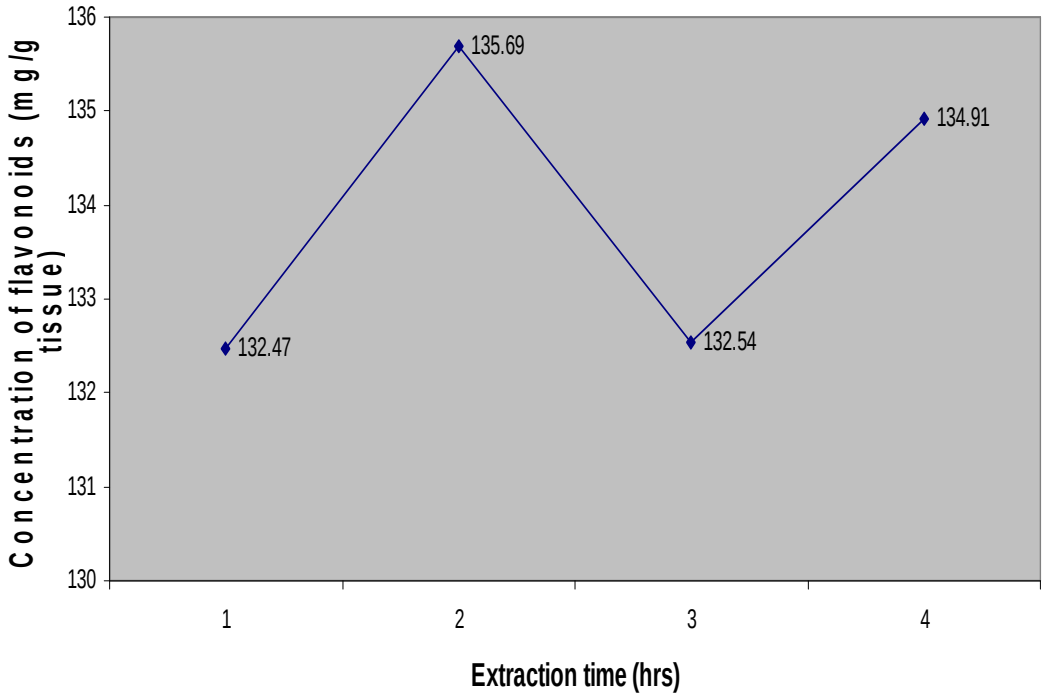


**Figure 2:** Effect of different extraction time.

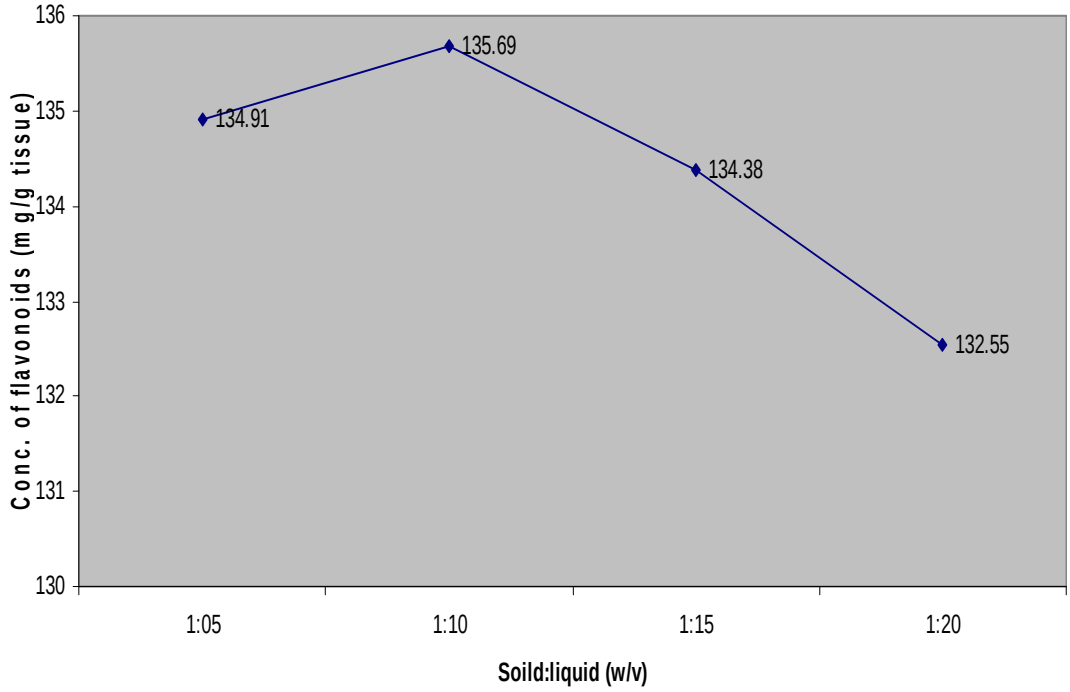


**Figure 3:** Effect of solid: liquid (w/v) in the extraction of flavonoids. Conc.: Concentration.

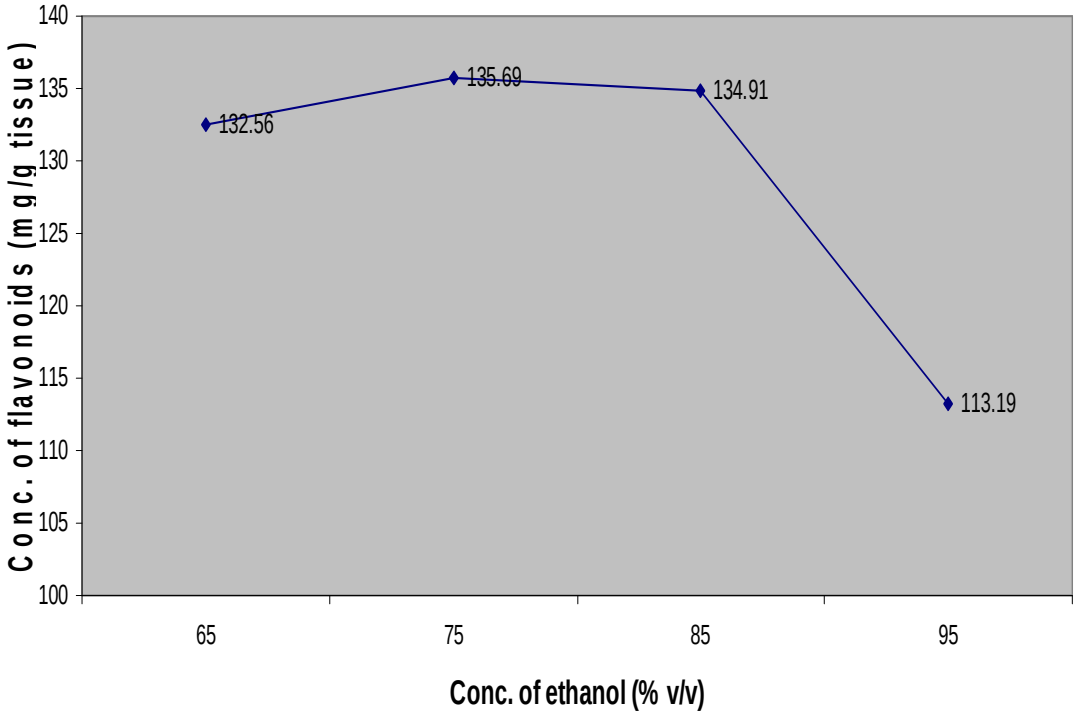


**Figure 4:** Effect of ethanol on the extraction of flavonoids. Conc.: Concentration.

**Table 1:** Factors for the extraction of flavonoids.

| Levels | A | B | C | D | E |
|---|---|---|---|---|---|
| | Temperature (ºC) | Extract time (hrs) | Solvent (%) | Solid : Liquid (W:V) | No. of extraction |
| 1 | 55 | 1 | 65 | 1:5 | 1 |
| 2 | 65 | 2 | 75 | 1:10 | 2 |
| 3 | 75 | 3 | 85 | 1:15 | 3 |
| 4 | 85 | 4 | 95 | 1:20 | 4 |

**Table 2:** $L_{16}$ orthogonal design of experiment (Chen et al., 2007).

| Experiments | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 2 | 3 | 4 |
| 2 | 1 | 2 | 1 | 4 | 3 |
| 3 | 1 | 3 | 4 | 1 | 2 |
| 4 | 1 | 4 | 3 | 2 | 1 |
| 5 | 2 | 1 | 1 | 1 | 1 |
| 6 | 2 | 2 | 2 | 2 | 2 |
| 7 | 2 | 3 | 3 | 3 | 3 |
| 8 | 2 | 4 | 4 | 4 | 4 |
| 9 | 3 | 1 | 3 | 4 | 2 |
| 10 | 3 | 2 | 4 | 3 | 1 |
| 11 | 3 | 3 | 1 | 2 | 4 |
| 12 | 3 | 4 | 2 | 1 | 3 |
| 13 | 4 | 1 | 4 | 2 | 3 |
| 14 | 4 | 2 | 3 | 1 | 4 |
| 15 | 4 | 3 | 2 | 4 | 1 |
| 16 | 4 | 4 | 1 | 3 | 2 |

ethanol acts as a potent modifier of cell membrane properties, especially in altering the permeability nature for solutes. Ethanol interacts with the flavonoids probably through non-covalent interactions and promotes a rapid diffusion into the solution (Luque de Castro and Tena, 1996). Various concentration of ethanol used exhibited different effect in changing the fluid polarity and thus has diversed effect on the solubility enhancement of the flavonoids (He Guo-qing et al., 2005). The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In this study, the results indicated 75% ethanol was found to be optimal for extracting the flavonoids.

**Effect of No. of extractions on flavonoids**

The contents of raw flavonoids extract increases with the number of extractions. Obviously, when the number of extraction times increases, the yield of the respective bioactive principle may be increased (Chen et al., 2007). In this investigation, the raw flavonoids content was increased by 2 times of the extraction.

**Optimization of flavonoids extraction using $L_{16}$ orthogonal design**

The parameters and the orthogonal design of experiment for the extraction of flavonoids were given in the Table 1 and Table 2. The results were made in the form of range analysis and one way ANOVA by Sigmastat 3.5 software. The results were depicted in Table 3 and Table 4. The order of the effect of factors on flavonoids extraction was A>D>E>B>C. The temperature parameter was observed to possess a greatest effect on the extraction procedure and the material ratio was found to be a secondary parameter even though it was not proved to be a significantly different at 5% level. The other factors such as solvent (%), extraction duration and no. of extractions may not play a vital role in extracting the flavonoids to a higher yield.

**Table 3:** Experimental results and range analysis.

| Experiments | A | B | C | D | E | Flav. (mg/g) |
|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 2 | 3 | 4 | 113.19 |
| 2 | 1 | 2 | 1 | 4 | 3 | 108.48 |
| 3 | 1 | 3 | 4 | 1 | 2 | 125.14 |
| 4 | 1 | 4 | 3 | 2 | 1 | 77.96 |
| 5 | 2 | 1 | 1 | 1 | 1 | 132.47 |
| 6 | 2 | 2 | 2 | 2 | 2 | 135.69 |
| 7 | 2 | 3 | 3 | 3 | 3 | 86.34 |
| 8 | 2 | 4 | 4 | 4 | 4 | 46.23 |
| 9 | 3 | 1 | 3 | 4 | 2 | 69.76 |
| 10 | 3 | 2 | 4 | 3 | 1 | 84.06 |
| 11 | 3 | 3 | 1 | 2 | 4 | 50.40 |
| 12 | 3 | 4 | 2 | 1 | 3 | 134.91 |
| 13 | 4 | 1 | 4 | 2 | 3 | 111.89 |
| 14 | 4 | 2 | 3 | 1 | 4 | 113.02 |
| 15 | 4 | 3 | 2 | 4 | 1 | 132.56 |
| 16 | 4 | 4 | 1 | 3 | 2 | 134.38 |
| $K_1$ | 53.2 | 64.8 | 84.6 | 104.7 | 90.2 | |
| $K_2$ | 63.3 | 80.4 | 85.3 | 80.5 | 62.8 | |
| $K_3$ | 94.8 | 66.4 | 77.7 | 82.7 | 82.3 | |
| $K_4$ | 109.9 | 88.8 | 73.5 | 53.3 | 85.9 | |
| $k_1$ | 13.3 | 16.2 | 21.2 | 26.2 | 22.6 | |
| $k_2$ | 15.8 | 20.1 | 21.3 | 20.1 | 15.7 | |
| $k_3$ | 23.7 | 16.6 | 19.4 | 20.7 | 20.6 | |
| $k_4$ | 27.5 | 22.2 | 18.4 | 13.3 | 21.5 | |
| ***R*** | ***14.2*** | ***6.0*** | ***2.8*** | ***12.9*** | ***6.9*** | |

**K** = Values obtained from individual factors (Temperature, Ext. time etc.,)**, R** = Rank.

**Table 4:** One way ANOVA levels.

| Levels | Sum of square | Degrees of freedom | Mean square | F-value |
|---|---|---|---|---|
| A | 2955.58 | 3 | 985.19 | 2.96 |
| B | 431.13 | 3 | 143.71 | 0.15 |
| C | 4318.01 | 3 | 1439.33 | 2.23 |
| D | 3272.56 | 3 | 1090.85 | 0.15 |
| E | 3052.92 | 3 | 1017.64 | 1.34 |
| | | 15 | | |

## Conclusion

It was concluded that by using $L_{16}$ orthogonal design of experiment the optimal conditions for the extraction of flavonoids was proved to be at 65 ºC, 2 hrs extraction duration, 75% ethanol, 1:10 material ratio and 2 times of extraction. The results proved that the temperature was found to be a major factor that affects the extraction procedure of flavonoids. Moreover, the TLC results of the optimized extracts were proved to contain quercetin and rutin related compounds. In future, further work may be attempted and extended for purifying the flavonoids and elucidating their structures which may be responsible for various applications.

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