

Antioxidant Activity of Melinjo Ketan (*Gnetum gnemon* L., 'Ketan') Seed Extract at Various Ripening Stages and Ethanol Solvent Concentration

Bambang Kunarto^{#*}, Sutardi Sutardi^{*}, Supriyanto^{*}, Chairil Anwar⁺

[#]Department of Agricultural Product Technology, Faculty of Agricultural Technology, Semarang University, Semarang 50196, Indonesia.

E-mail: bambangkun@usm.ac.id

^{*}Department of Food Science, Faculty of Agricultural Technology, Gadjah Mada University, Bulaksumur Yogyakarta 55281, Indonesia.

E-mail: sutardi@ugm.ac.id and supriyanto@ugm.ac.id

⁺Department of Chemistry, Faculty of Mathematics and Natural Science, Gadjah Mada University, Yogyakarta 55281, Indonesia,

Email: irilwar@yahoo.com

Abstract— Melinjo seeds (*Gnetum gnemon* L.) have the potency as antioxidants due to their bioactive compounds such as polyphenols, flavonoids, tannins, and resveratrol. The study was conducted to determine the effect of the ripening stages of Melinjo ketan variety seeds and ethanol solvent concentration on total of phenolic, flavonoid also resveratrol content, and antioxidant activity of Melinjo seed extract. The ripening stages of Melinjo seeds used were immature, semi-mature and mature. The ethanol solvent concentrations were 20%, 50% and 80%. Samples of dried melinjo seeds were macerated in ethanol (1: 5) at room temperature for 2 days. The extract was filtrated, evaporated and freeze-dried. This study reported that the ripening stages of melinjo seeds and ethanol solvent concentrations had effect on the total of phenolic, flavonoid, resveratrol content, and antioxidant activity of melinjo seed extract significantly ($p < 0,05$). Mature melinjo seeds macerated using 50% ethanol solvent produced extract with the highest antioxidant activity. It had the total phenolic content of 7.033 ± 0.06 mg GAE/g, total flavonoids content of 335.04 ± 1.32 mg CE/100 g, resveratrol content of $1.55 \pm 0.3\%$, DPPH radical inhibition of $66.07 \pm 0.38\%$, and reducing power ($80.26 \pm 0.06\%$). These studies showed that the antioxidant effect of Melinjo ketan extract is influenced by the level of maturity and solvent concentrations of ethanol that can be an alternative of antioxidant sources. It is recommended to use Melinjo ketan seeds macerated with 50% ethanol solvent.

Keywords— antioxidant; melinjo ketan; resveratrol; ripening stage.

I. INTRODUCTION

Melinjo (*Gnetum gnemon* L.), including the family of Gnetaceae which is spread in Southeast Asia, especially in the territory of Indonesia. Mostly in Indonesia, melinjo's seeds and rinds are consumed as vegetables or snacks. Tatefuji *et al.* [1] states that melinjo seeds are not toxic and can be consumed in the long term. Melinjo seeds are oblong and have the size of 2.5 x 1.5 cm. There are several varieties of melinjo, which differ in the size of the fruit [2]. Melinjo ketan is one of the melinjo varieties. It has oval shaped. Its size is larger than melinjo kerikil, but smaller than melinjo gentong.

Previous studies investigated that melinjo seeds contain bioactive compounds such as polyphenols, flavonoids, tannins, and resveratrol. It indicates that melinjo seeds have the potential to be antioxidants source. Resveratrol (3,5,4-

trihydroxystilbene) is the compounds formed naturally from the types of polyphenols and phytoalexin [3]. Resveratrol has been reported to have antioxidant activity, anti-aging effects, anti-carcinogenic activity [4], hepato-protection [5], cardioprotection and neuroprotection [6,7], anti-inflammatory activity [8], anti-obesity [9], and diabetes prevention activities [10]. Resveratrol is a group of hydroxyl having positions of 3, 4 'and 5, aromatic rings and there are double bonds in resveratrol molecules, thus having a very strong ability in avert of free radicals [11]. Gerzon *et al.* [12] states that the elimination of the hydroxyl group or its suspension by cluster – OCH₃ results in the loss of antioxidant properties in compounds. Based on the efficacy, the use of resveratrol as a bioactive agent in food and pharmaceuticals has been a concern [13]. It is expected that Melinjo ketan seed extract can be applied to food as an alternative to substitute synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxytoluene

Hydroxyanisol, propyl error and tert-butyl hydroxyl Quinone, because Synthetic antioxidants in food are not safe for consumers [14] [15].

The level of fruit ripening affects antioxidant activity as reported in several studies. The phenolic profile and antioxidant activity of lemons vary depending on different ripening stages [16]. The difference in harvest time of Pinot Noir and Merlot (*Vitis vinifera* L. cv.) grape berries significantly affects phenolic and physicochemical content [17]. Generally, the process of fruit ripening is consisting of changes of biochemical compound and primary & secondary metabolism, which produce phytochemicals and nutrients that have the potency to improve health [18]. Blank *et al.* [19] states that the total phenolic, flavonoids and antioxidant activity of *Bunchosia glandulifera* seeds increased according to the level of its efficacy. The concentration of solvent influences the antioxidant activity as well. The general principle of extraction using solvents is “like dissolve like”, that solvents extract phytochemicals only that have the same polarity as solvent [20].

The efficacy of melinjo ketan extraction based on the ripening stage is still unclear. Our study aimed to investigate the effect of ripening stages and ethanol solvent concentration to the phenolic total, flavonoid total, resveratrol content, and melinjo ketan seed extract antioxidant activity.

II. MATERIAL AND METHOD

A. Materials

Melinjo ketan seeds were obtained from Limpung district, Batang Regency, Central Java, Indonesia. Melinjo were harvested manually from several trees randomly. The fruits were selected become three stages of maturation according to age after flowering and external colour. Immature melinjo had green rind and was harvested at 60 days after flowering. Semi-mature melinjo had orange rind and was harvested at 68 days after flowering. While mature melinjo had red rind and was harvested at 75 days after flowering. The physical characteristics of melinjo ketan variety at various levels of maturity are shown in Table 1 and Figure 1.

TABLE I
PHYSICAL CHARACTERISTIC OF MELINJO KETAN AT DIFFERENT RIPENING STAGES

Characteristic	Immature	Semi-mature	Mature
Age after flowering (days)	60	68	75
Peels color	Green	Orange	Red
Length (cm)	2,70±0,01	2,71±0,05	2,72±0,01
Width (cm)	1,20±0,01	1,21±0,01	1,24±0,05
Weight (g)	4,03±0,06	4,07±0,06	4,133±0,06

B. Sample Preparation

Melinjo ketan seeds were separated mechanically from the skin and shell, then freeze-dried. The freeze-dried melinjo seeds were reduced in size using a grinder and sifted to powder that passed the 40 mesh sieve. The powder sample was packaged in an airtight polyethylene plastic bag and stored until used at 4 °C.

C. Maceration

Extraction of melinjo ketan seeds powder was carried out by maceration conducted to Kato *et al.* [21] with some modifications. One hundred gram of melinjo seed powder samples (immature, semi-mature and mature) were macerated in 500 ml ethanol at room temperature for 2 days. The concentrations of ethanol solvent used were 20%, 50% and 80%. Melinjo seed extract was filtered, then the filtrate was evaporated in vacuum until viscous. The viscous extract

of melinjo seeds was freeze-dried at -40°C and 10⁻¹ torr. Furthermore, the freeze-dried extract of melinjo seeds obtained was stored in a dark place at 4 °C until it is ready to used for analysis.

D. Analysis of Total Phenolic Content

The melinjo seeds extract was analyzed to measure the total phenolic content by using the Folin-Ciocalteu assay according to Dewanto *et al.* [22] with few modifications. The 125 µl of melinjo seed extract sample of were dissolved in 500 µl of distilled water then add 125 µl of Folin-Ciocalteu reagent solution until homogenized. Add 1.25 ml of Na₂CO₃ 7%, and distilled water was added to a volume of 3 ml. The absorbance measurement at 760 nm was carried out after incubation in the dark room for 90 minutes. The total phenolic content was expressed as mg Gallic Acid Equivalent (GAE) per gram of dry sample.



Fig. 1 Ripening stage of melinjo ketan

E. Total Flavonoid Content Analysis

The total flavonoids of melinjo ketan seed extract were determined based on the method used by Zhishen *et al.* [23] and Bhat and Yahya [24]. The melinjo seed extract sample of 1 ml was added with four ml of distilled water. At zero time, there was an addition of 0.3 ml of NaNO₂ 5% (w/v). After 5 minutes, the solution was adding by 0.3 ml of AlCl₃ 10% (w/v). The two ml of 1 M NaOH were added in the 6th minute and the volume until 10 ml by addition of distilled water. This mixture was vortex and the absorbance was analyzed by using a UV-visible spectrophotometer at 510 nm. The results obtained were stated as mg Catechin Equivalent (CE) per 100 g of dry sample.

F. Determination of Resveratrol Content

A total of 0.1 ml of melinjo seed extract was placed in the microtube, then added with 0.9 ml of ethanol. The mixture was homogenized using vortex for 2 minutes and centrifuged for 5 minutes. The 20 µl of extract was spotted into silica gel plate F254 and eluted in the mobile phase according to the mobile phase used by Liu *et al.* [25], namely toluene: ethyl acetate: acetic acid (15: 3: 1). The density was reading by TLC-scanner.

G. DPPH Radicals Inhibition

Antioxidant activity of melinjo seed extract was determined using DPPH assay according to Savic-Gajig *et al.* [26]. Melinjo seed extract was added by 1 ml of DPPH solution. After mixing, it were incubated at room temperature for 30 minutes in less light conditions. The absorbance of the samples were quantified at 517 nm. Antioxidant activity was stated as DPPH radicals inhibition (%) and analyzed by using the equation 1:

$$\text{Inhibition of DPPH radicals (\%)} = \frac{(A_c - A_s)}{A_c} \times 100 \quad (1)$$

where A_c and A_s are absorbance of the control solution and absorbance of the samples treated with DPPH radicals, respectively.

H. Reducing Power

Reducing power was based on the method described by Ozcoy [27] with some modifications. The absorbance of the solution was measured at 700 nm. The percentage of reducing power samples compared to the standard (ascorbic acid) was calculated using the following equation 2.

$$\text{Reducing power (\%)} = \left[1 - \left(1 - \frac{A_s}{A_c} \right) \right] \times 100 \quad (2)$$

where A_c and A_s are standard absorbance and absorbance of the, respectively.

I. Statistical Analysis

The research design was used a randomized design with two factors and three repetitions. These factors were the ripening stages of melinjo seeds (immature, semi-mature, mature) and the concentration of ethanol solvents (20%, 50%, 80%). The results were represented as mean values and standard deviations (SD). The statistical data analysis was performed by analysis of variance (ANOVA). If there were differences between treatments, then the analysis was continued with Duncan's multiple range test (DMRT) at the level of 5%.

III. RESULT AND DISCUSSION

A. Analysis of Total Phenolic Content

The total phenolic content (TPC) of melinjo ketan seed extract at three different maturity levels (immature, semi-mature, and mature) and macerated with various ethanol concentrations is shown in Figure 2. The maturity level of melinjo seeds and ethanol solvents significantly affected ($P < 0,05$) to the total phenolic content of the extract. Melinjo seeds with a higher maturity stage showed an increase in total phenolic extract. This is supported by previous study. Yang *et al.* [28] stated that maturity increases the total phenolic content of noni fruit. Mahmood [29] also reported that the more mature blueberries and mulberries showed an increasing total phenolic content.

The immature melinjo ketan seed extract had a lower total phenolic content than semi-mature and mature melinjo seed extract. It happened in the extract macerated at ethanol concentrations of 20%, 50% and 80%. The total phenolic content of melinjo seed extracts macerated using 20% ethanol solvent were 1.70 ± 0.006 , 1.83 ± 0.05 , 4.13 ± 0.16 mg GAE/g, respectively for immature, semi-mature and mature. Total phenolic content of immature, semi-mature and mature melinjo ketan seed extract macerated using 80% ethanol solvent were 3.24 ± 0.05 , 3.41 ± 0.31 , and 6.22 ± 0.57 mg GAE/g, respectively.

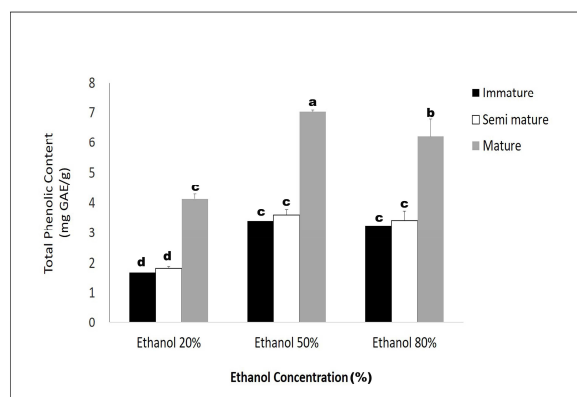


Fig. 2 Ripening stage of melinjo ketan seed extract

Interestingly, mature melinjo ketan seed extract macerated using 50% ethanol solvent had the highest total phenolic content production were 7.033 ± 0.058 mg GAE/g extract. The maturation was increased enzyme activity for the Biosintesa phenol. The total increase of phenolic content in the mature stage can be caused by the higher production of phenylalanine ammonialyase [30]. The total phenolic content of melinjo seed extracts in this study are different from previous study. Bhat and Yahya [24] reported that TPC evaluated in melinjo ethanol extract was 15.1 ± 2.19 mg GAE/100g. In other study, Wazir *at al.* [31] reported that TPC melinjo seed extract ranged from 1.15 to 6.49 mg GAE/g FDW. The difference of total phenolic occurred by several factors, including varieties, age, place and environmental conditions of melinjo growth and its cultivation.

B. Total Flavonoid Content

Maceration of melinjo ketan seeds with different ripening stages using various ethanol concentrations as solvents was produced extracts with significantly different of flavonoid ($p < 0.05$). The flavonoid content of melinjo seed extract also

increased along with the maturity of melinjo seeds (Figure 3). The total flavonoid content of immature, semi- mature and mature melinjo ketan variety seed was extracted using 20% ethanol solvent were 199.21 ± 0.12 , 200.40 ± 0.26 , and 251.78 ± 2.27 mg CE/100g, respectively. While the total flavonoid content of melinjo extracts macerated using 80% ethanol solvents were 218.31 ± 0.29 , 288.88 ± 1.77 , and 296.79 ± 1.94 mg CE / 100 g, respectively for the immature, semi-mature, and mature. However, the highest total flavonoid content (335.04 ± 1.35 mg CE/100g) was obtained from mature melinjo ketan variety seeds macerated using 50% ethanol.

The results of this study are in accordance with Bettaieb-Rebey [32] which showed that the more mature cumin seed (*Cuminum cyminum* L.), the higher the flavonoids in it. Mahmood *et al.* [29] also stated that total flavonoids had increasing maturity in strawberry and mulberry from Pakistan. Moreover, Duma *et al.* [33] described bioactive compounds of tomatoes at various ripening stages and observed the increase in total flavonoids as maturity developed.

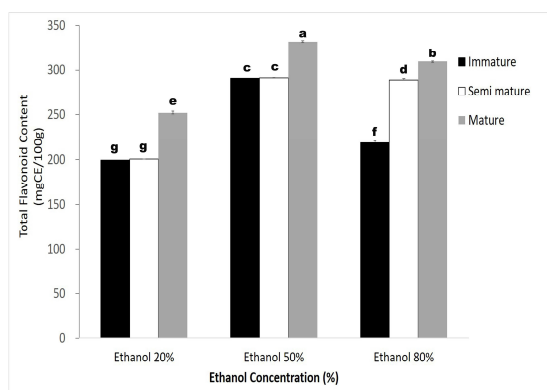


Fig. 3 Total flavonoid content of melinjo ketan seed extract

C. Resveratrol Content

Resveratrol is synthesized through the phenylalanine pathway. The stilbene synthase (STS), phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), and coumaroyl-CoA ligase (4CL) have important roles in the synthesis [34]. The synthesis of resveratrol is increased by the presence of wounds, infections, or stress. Various

concentrations of ethanol solvents for maceration of melinjo seeds with different ripening stages showed an effect on the content of resveratrol significantly ($p < 0.05$). The resveratrol content of immature, semi-mature and mature melinjo seeds macerated using 20% ethanol were 0.14 ± 0.004 , 0.17 ± 0.06 , and $0.54 \pm 0.02\%$ (dry weight).

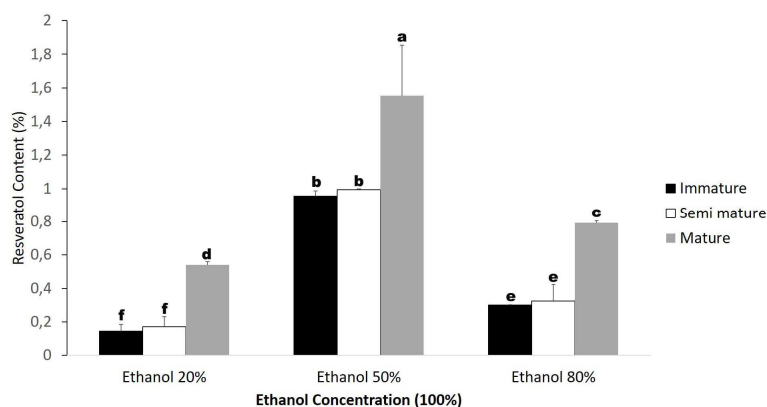


Fig. 4 Resveratrol content of melinjo ketan seeds extract

Melinjo ketan seed extract using 80% ethanol had the resveratrol content of 0.3 ± 0.001 , 0.32 ± 1.00 , and $0.79 \pm 0.01\%$ (dry weight), respectively for immature, semi-mature and mature melinjo seeds. The highest resveratrol content ($1.55 \pm 0.3\%$) was investigated in melinjo seeds extracts using 50% ethanol (Figure 4). This study is consistent with Wang *et al.* [28] that mature strawberries seeds and pulp contained more resveratrol than immature strawberries. In addition, the use of 50% ethanol in maceration produced the highest resveratrol in melinjo seed extract compared with the use of 20% and 80% ethanol concentrations. This is in accordance with Kato *et al.* [21]. They extracted and isolated stilbenoid from melinjo seeds using 50% ethanol to obtain gnetin L, gnetin C, gnetin A, C, D, and resveratrol.

D. Antioxidant Activity

Plants have many groups of compounds that have antioxidant properties. Therefore determining antioxidant activity is difficult and requires a long time [24]. There are many developed method to determine antioxidant activity in plant material [36]. The methods should be capable of measuring the antioxidants to inhibit free radicals by lipid

peroxidation or chelating metals inhibition. In this study, antioxidant activity assay of melinjo ketan variety seed extract was carried out using two commonly used methods of radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power. In the DPPH assay, a reaction occurs between antioxidants and free radicals (DPPH) which results in a reduction in DPPH concentration. It leads to color changes which can be measured at certain wavelengths.

In this study, DPPH radicals inhibition increased according to the maturity of melinjo seeds. DPPH radicals inhibition of melinjo ketan variety seed extract using 20% ethanol were 42.54 ± 0.024 , 43.95 ± 0.41 , and $45.37 \pm 0.29\%$, respectively for immature, semi-mature, and mature melinjo seeds. The immature, semi-mature and mature melinjo ketan variety seed using 80% ethanol had DPPH radical inhibition of 45.43 ± 0.29 , 42.19 ± 0.88 , and $49.17 \pm 0.89\%$, respectively. Interestingly, the highest DPPH radicals inhibition was obtained from mature melinjo seed macerated using 50% ethanol (Figure 5). This study indicated that 50% ethanol solvents tended to be more effective in extracting antioxidant compounds

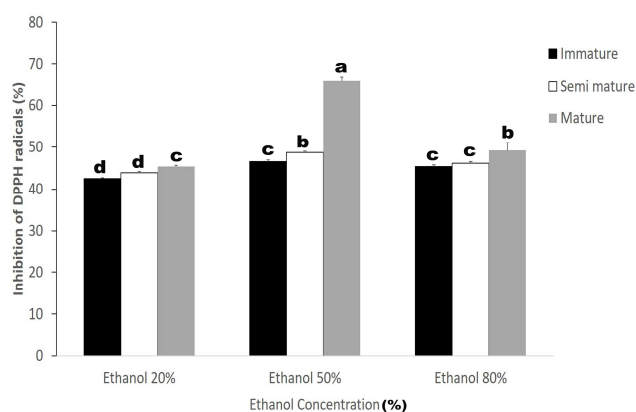


Fig. 5 Inhibition of DPPH radicals of melinjo ketan seed extract

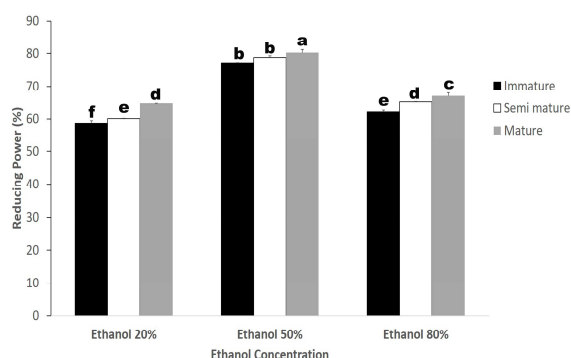


Fig. 6 Reducing power of melinjo ketan seed extracts

The reducing power method is according to the ability to reduce Fe^{3+} ions to Fe^{2+} . The antioxidant activity is presented by the ability of melinjo seed extract to reduce these compounds. Reducing power from various extracts and standards (ascorbic acid) was using by potassium ferricyanide reduction. The highest reducing power ($80.26 \pm$

0.06%) was obtained from mature melinjo ketan variety seed extract macerated using 50% ethanol as a solvent (Figure 6).

The antioxidant effect from extract of melinjo ketan variety was related to the total of phenolic, flavonoid, and resveratrol content. Increased of total phenolic content, total flavonoid content and resveratrol content in mature melinjo

seed extract was followed by the increased of antioxidant activity. This is consistent with previous findings reported that phenolic compounds have high antioxidant capacity [37].

Atanassova *et al.* [38] found that flavonoids are responsible for the ability to counteract free radicals and chelate metal ions. The antioxidant activity of Polygonum equisetiforme extract also showed a relatively similar pattern with the total phenolic and flavonoids content [39]. Li *et al.* [40] conducted a study of traditional Chinese herbal medicines. They investigated that flavonoids content in medical plants may be important phytochemical compounds as antioxidant. According to the previous study, the resveratrol has the ability as an antioxidant [21, 41].

E. FTIR Spectroscopy

The infrared spectrum represents a functional group. The infrared spectrum of melinjo ketan variety seed extracts at different maturity level had similarities. However, mature melinjo ketan variety seed macerated using 50% ethanol had a higher absorption intensity. The relationship between wave number, vibration band, and chemical compound is shown in Table 2. The infrared spectrum of melinjo seed extract using ethanol solvent is shown in Figure 7.

TABLE II
INFRARED VIBRATIONAL BANDS OF MELINJO KETAN SEEDS EXTRACT AT DIFFERENT MATURITY STAGES

Wave number (cm ⁻¹)	Vibration band/group	Chemical compound
3297.31-3302.04	OH stretch, H bonded	Phenols, alcohols
2956.92-2959.43	C-H (Asym.)	Alkanes
	O-H Stretch	Carboxylic acid
840.99-2847.85	C-H stretch (sym.)	Alkanes
1634.93-1644.86	C=O stretch (carbonyl)	Flavonoids, polyphenols
	C=C stretch	Aromatics
1407.88-1451.21	C-C stretch	Aromatics
1233.76-1265.14	C-N stretch	Aliphatic Amines
1015.01-1015.10	C-O stretch	Alcohols, esters, carboxylic acids
	C-OH stretch	Secondary alcohols

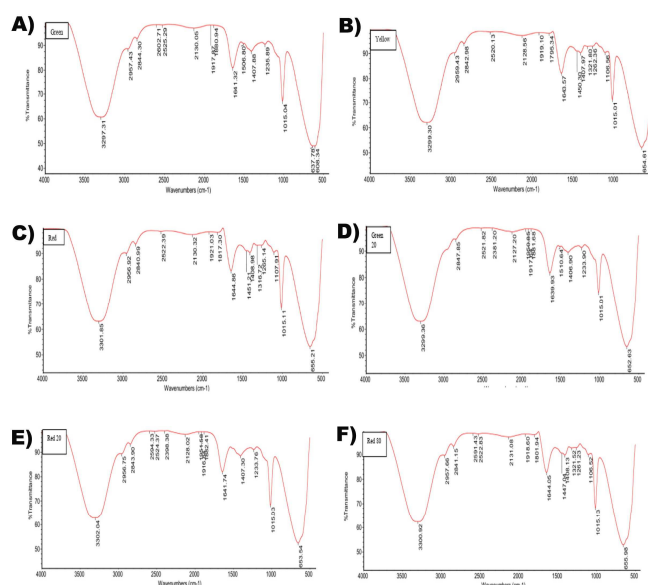


Fig. 7 The infrared spectra of melinjo ketan seed extract macerated using ethanol. A) Green (50% ethanol), B) Orange (50% ethanol), C) Red (50% ethanol), D) Green (20% ethanol), E) Red (20% ethanol), F) Red (80% ethanol).

IV. CONCLUSIONS

Melinjo ketan seeds may be an alternative of antioxidant sources. These results indicate that the antioxidant activity of melinjo ketan was influenced by the level of maturity and solvent concentrations of ethanol. Mature melinjo seeds macerated using 50% ethanol solvent had the highest antioxidant activity.

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