ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at www.jgpt.co.in

RESEARCH PAPER

# Antioxidant Activity and Immunomodulator of Indonesia Black Rice(*Oryza sativa* L. *indica*) Extract

**Fadjar Kurnia Hartati[1,4]*, Simon Bambang Widjanarko[2], Tri Dewanti Widyaningsih[2], Muhaimin Rifa'i[3]**

*[1]Post Graduate Program, Faculty of Agricultural, Brawijaya University, Malang, Indonesia.*

*[2]Department of Food Science and Technology, Faculty of Agricultural Technology, Brawijaya University, Malang, Indonesia.*

*[3]Biology Department, Faculty of Mathematic and Natural Sciences, Brawijaya University, Malang, Indonesia.*

*[4]Department of Food Technology, Faculty of Agricultural, Dr. Soetomo University, Surabaya, Indonesia.*

***Corresponding Author: Fadjar Kurnia Hartati**

## Abstract

The aim of this study to explore the foodstuffs potentially as an immunomodulator for use of synthetic immunomodulatory in the long term, in addition to considering also has negatively affect to the organs. The materials normally used as an immunomodulator is a substance containing phenol and flavonoid that has antioxidant activity. One of them is Indonesia black rice. This study was performed by in vitro test so that the samples that tested were in the extract form. Extracts were tested were ethanol and water extract of black rice. The use of water as a solvent extract because the sample is food, While ethanol is also used because ethanol is not toxic and the compounds present in black rice are also polar so polar that they can be used in dissolving polar and semi-polar compounds. Each extract was measured levels of phenol, antioxidant activity and its effects on lymphocyte proliferation (as an organ where immune cells produced). The treatment was health control, pain control, 50,100 and 200µg/ml. The results showed that ethanol extract of black rice and water containing phenol compound and has activity as an antioxidant, it also very potential as immunomodulators because the extract can increase lymphocyte proliferation.

**Keywords:** *immunomodulator, ethanol, water, Indonesia black rice.*

## Introduction

Immunomodulator is a substance that can help the body to optimize the function of the immune system. The immune system is the main system functions in the defense of the body where most people are prone to disorders of the immune system. When the immune system isn't working optimally, the body will be susceptible to the disease[1].

There is a relationship between the antioxidants in the diet and immune function so it takes extra antioxidants from outside the body in the form of food intake [2]. The use of synthetic immunomudulator especially in the long term, in addition to considering also adversely affects the kidneys and other organs [3]. The food-stuffs containing polyphenols compound is a source of antioxidants, which are abundant in fruits, vegetables, grains and other food products. Polyphenols are effective antioxidants because of the hydroxyl groups that have the ability to provide electrons, so it can eliminate free radicals [4-5]. Polyphenols and flavonoids compound as antioxidants also shown to enhance the immune response [6].
The way to determine the immunomodulatory activity of substance is by testing the cell proliferative activity of lymphocytes as the immune cells produced [7].

The food that has potential as an antioxidants and immunomodulators is black rice (*Oryza sativa* L. *indica*) because it contains anthocyanins, flavones and flavonols, carotenoids and oryzanol compound [8]. Some black rice research more on anthocyanins, such as black rice anthocyanin extracts reported could inhibit

liver cancer cells [9], decrease the cholesterol levels, triglisedrida, LDL (Low Density Lipoprotein) and increase HDL (High Density Lipoprotein) and very potential for cardiovascular therapy [10-12], can improve the function of spleen, liver, gastric and intestines, also as a hematopoietic agents in the pharmacy [13], prevent atherosclerosis [14], anti-inflammatory [15], in the other take advantage of black rice bran as a natural dye [16] and other research observed the effects of black rice bran extract as an antioxidant and hepatoprotective [17].

But there is no one has studied the activity of ethanol and water extract as an antioxidant and immunomodulatory. The use of aqueous extracts for health reasons, while the ethanol extract as a comparison. There are many polar compounds that dissolved in ethanol as a bioactive compound [18].

## Method

## Chemicals

Chemicals used for analysis is pro analysis Merck (Germany). While the standard use of chemicals pro analysis from Sigma-Aldrich (German); PBS, RPMI 1640 FBS, anti-CD3, LPS, penicillin and streptomycin, STZ, 2-Mercaptoethanol (Biowest, USA).

### Plant Material

Black rice (*Oryza sativa L.*) used Wojalaka varieties from Kepanjen, Malang, East Java, Indonesia. These varieties included in the Indica group [19].

### Extracts Preparation

The black rice is dried and pulverized (± 80 mesh) into simplicia. The ethanol extract derived from simplicia that macerated in ethanol 96% (1:10 (g/ml) for 24 hours, while the water extract derived from simplicia that macerated with distilled water for 12 hours with stirring a few times. After that filteredfor separating the pulp and the filtrate. And than the ethanol filtrate was evaporated using rotary evaporator with temperature of 40±5 °C until thick and dried with a freeze dryer then weighed to determine the yield. The filtrate water was drained with freeze dryer (-40 °C).

### Phytochemical Analysis

### Total Phenolic Content

The total phenol content assay of ethanol and water extract from black rice using Folin-Ciocaltea[20] with Gallic acidas standard. 0.1 ml of extract added with 3.9 ml of distilled water and 0.5 ml Folin-Ciocalteu reagent (1:10 in distilled water). The solution was allowed stand for 3 minutes and then add 2 ml of 20% $Na_2CO_3$ and the absorbance values was measured by UV-spectrophotometer at 756.5 nm.

A calibration curve prepared from the absorbance value of gallic acid solution at a various concentrations. The content of total phenols in ethanol and water extract from black rice expressed as mg gallic acid equivalents (GAE)/g of dry extract.

### Antioxidant Activity

### Lipid Peroxidation Inhibition Activity

The ethanol and water extract from black rice was weighed as much as 10.0 mg added with ethanol p.a to 10 mL. The samples were prepared from a solution of ethanol and water extract of black rice as much as 4 mL with concentration 1 mg/mL, 4.1 mL of 2.52% oleic acid, 8 mL 0.02 M phosphate buffer and water as much as 3.9 mL were incubated at 40 °C and read every 24 hours until got a maximum absorbance of the negative control (oleic acid mixture, phosphate buffer, water and extract solvent). During incubation, 0.1 mL of the incubated samples awere taken and then added 9.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 0.02 M ferrous chloride were incubated for 3 minutes and then read on the 500 nmlength wave [21].

### Total Antioxidant Activity(TAA)

The total antioxidant activity of black rice extract (ethanol and aqueous) were evaluated by phosphomolybdenum method
[22]. The assay is based on the reduction of Mo (VI) - Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Different extracts of 10 µL each from the stock solution were dissolved in 90 µL distilled water and 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in 1.5 mL tubes. The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the solution of each reaction was measured at 695 nm against blank samples. Ascorbic acid (AA) was used as standard and the total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (mg AAE/g) of dw.

**Lymphocyte cell proliferation / CFSE assay [23]**

**Animals ExperimentalPreparation**

Animals that be used were Mus musculus (mouse) male Balb / C strain and pathogen free. The age of experimental animals be used were four weeks while STZ injection. The research procedure was approved by the Research Ethics Committee (Animal care and Use Committee) of Brawijaya University and expressed Eligible Ethics.

**Splenocyte Cell Culture**

The spleen was isolated from a health mice and mice models of diabetes mellitus (DM) then washed with phospho-buffered saline (PBS). Cells were isolated from the spleen by crushing it in PBS.

The homogenates were centrifuged at 2500 rpm, 10 °C for 5 minutes. Pellet cells labeled with CFSE 5 mL in 500 µL of PBS and then incubated for 10 minutes in the ice box. After that, coupled with 500 µL of FBS and centrifuged to remove CFSE residual that does not label cells. Pellets are resuspended in 1 ml of PBS and then centrifuged to remove FBS residual. Finally, the pellet resuspended in 1 ml of medium and then resuspended to remove any remaining PBS.

Cells grown in 48 culture well plate in 1 ml/well (3 billion/ml). That cells were incubated in a 5% $CO_2$ incubator at 37 °C for 5 days. After a period of incubation, cells were harvested by resuspanted of each treatment media to cell homogenate and move it into a 15 ml propylene tubes and then centrifuged at 2500 rpm at a temperature of 10 °C for 5 minutes, and continued to flowcytometry procedure.

**Flowcytometry**

Cells were incubated added by 300-500 µL of PBS. Each sample was transferred into the corresponding cuvettes flowcytometry for immediately running by flowcytometry. Flow cytometry analysis performed by using software CellQuestPro.

**Results and Discussion**

**Phytochemical Analysis**

The results of the total phenols and flavonoidsassay in ethanol and water extract of black rice can be seen in Table 1. The total content of phenol in ethanol extract (BE) is different from total phenol content in the water extract (BA). Total phenol in ethanol extract (95.58± 0.71) mg GAE/g is higher than water extract (58.42±0.23) mg GAE/g dw (Table 1)

**Table 1: Total phenol of black rice extract**

| Extract | Total phenolic content (mg GAE/g of plant extract) |
|---|---|
| BE | $95.58 \pm 0.71_b$ |
| BA | $58.42 \pm 0.23_a$ |

BE: Ethanol Extract; BA: Water Extract

A phenol compound as secondary metabolites in plant has a potential as antioxidants and may protect cells against oxidative damage. This is caused by the presence of hydroxyl groups in the phenol compound. Hydroxyl groups can serve as a contributor to the hydrogen atom when it reacts with radicals through electron transfer mechanism that inhibited oxidation processes [25-26].

Therefore poliphenol and flavonoids are widely used as anti-cancer material, antidiabet, neuroprotective, antimicrobial, hepato-protective, cardio protective and immunomodulatory [24].

**The Antioxidant Activity**

**Inhibition of Lipid Peroxidation Method / Ferric Thiocyanate Method (FTC)**

Lenoleat acid incubated until it be oxidize that used as a reactive compound / free radicals in the FTC method (Ferric Thiocyanate) to determine the ability of a material as antioxidant compound[27]. This is due to a variety of diseases including disruption of the immune system that caused by free radicals that can damage the unsaturated fatty acids, cell wall membrane, blood vessels, bases of DNA, and lipid tissue [28].

The strength of antioxidant activity in inhibiting the oxidation lenoleat acid in the extract can be seen in Table 2. The ethanol extract was 53.42±0.37; water extract was 44.16±0.29 while vitamin C amounted to 68.65±0.19, on the 6thday. Oxidation of fats into peroxide will oxidize $Fe^{2+}$ to $Fe^{3+}$. Furthermore, $Fe^{3+}$ will form a complex with SCN be red ferritiosianat.

**Table 2: Antioxidant capacity of extract black rice**

| Extract | Ferric Thiocyanate | Total antioxidant capacity |
|---|---|---|
| BE | 53.42 ± 0.37b | 132.38 ± 0.70b |
| BA | 44.16 ± 0.29a | 96.70 ± 0.30a |
| Vit C | 68.65 ± 0.19c | 140 + 0.05c |

BE: Ethanol Extract BA: Water Extract, Vitamin C

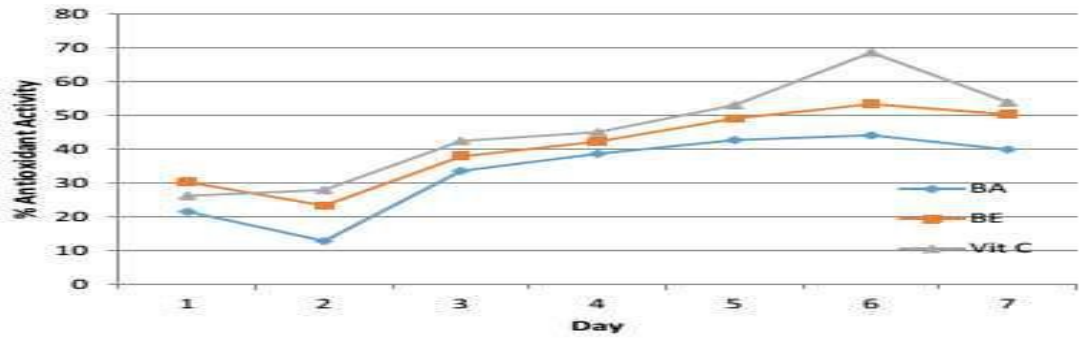



**Figure 1. Hike percent inhibition profile of lipid peroxidation within 7 days**

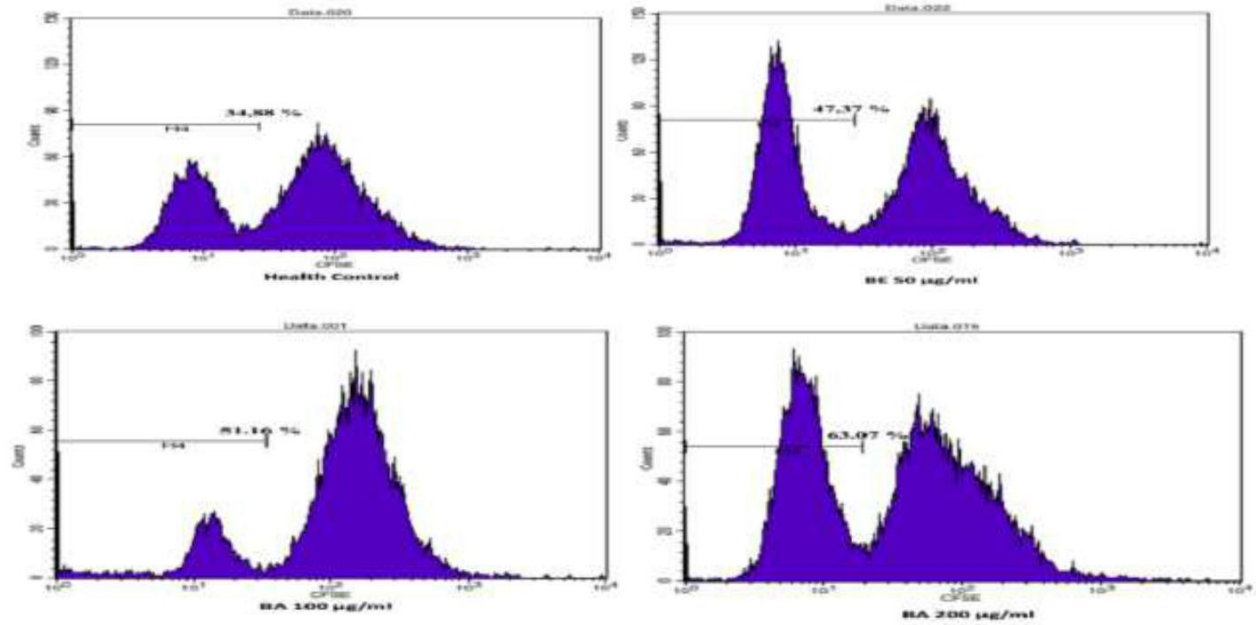
Phenolic and flavonoids compounds are the hydrogen donors for the free radicals formed during lipid peroxidation. Antioxidants can reduce hydroperoxy radical formation in the early phase of lipid peroxidation chain reactions through solving [29]. The low absorbance show high antioxidant activity due to radicals formed during lipid peroxidation forming a stable final product. Radicals formed relatively stable because of resonance and not easy to follow in a chain reaction [30].

## Total Antioxidant Activity

The determination results of the antioxidant activity total by using phosphor molybdenum methods can be seen in Table 2. The ethanol extract has greater antioxidant activity total (132.38±0.70) compared with water extracts (96.70±0.30). One indicator of antioxidant activity is the ability of an extract to donate electrons [31-32]. This is consistent with several studies showing that the antioxidant activity total related to levels of total phenols [33-34].

## Lymphocyte Cell Proliferation (CFSE assay)

Immuno competent cells labeled with CFSE showed higher cleavage activity when stimulated by administration of ethanol and water extract of black rice (Figure 2). Cells undergoing division located on the left at the peak flow cytometry analysis because it shows a decrease in CFSE luminescence.



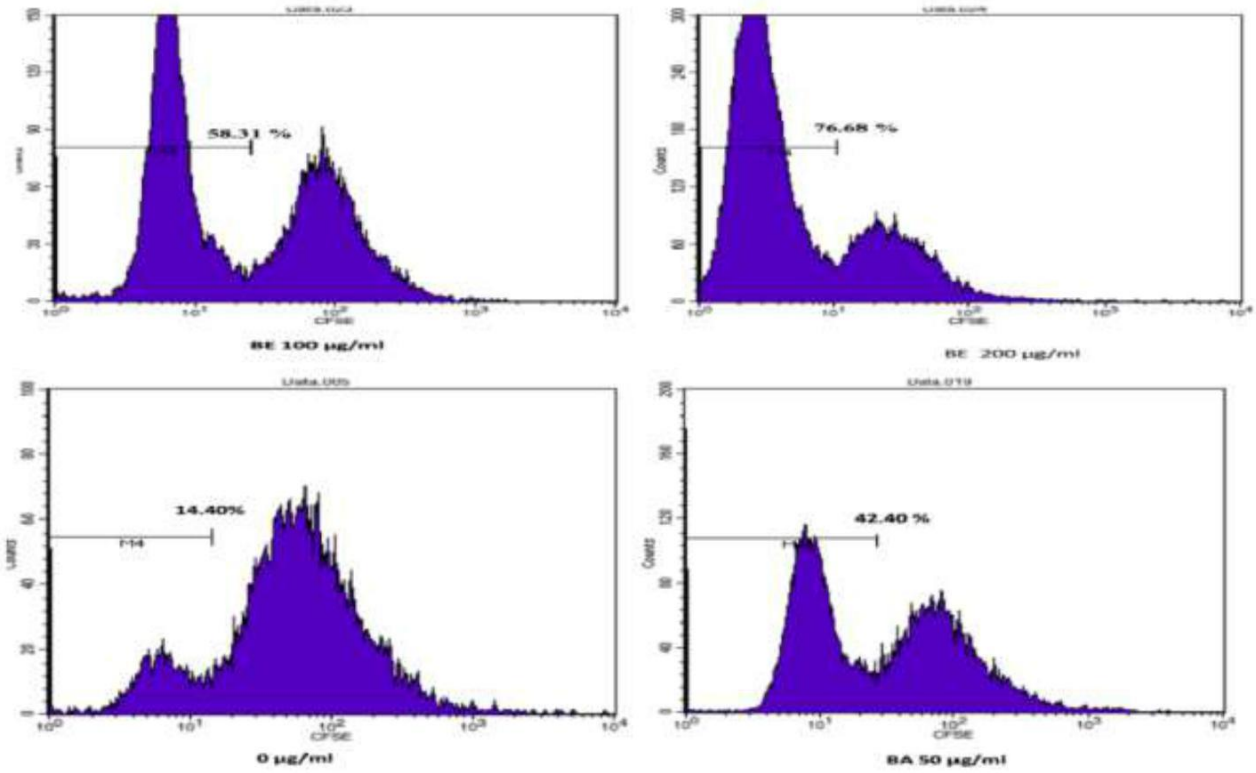
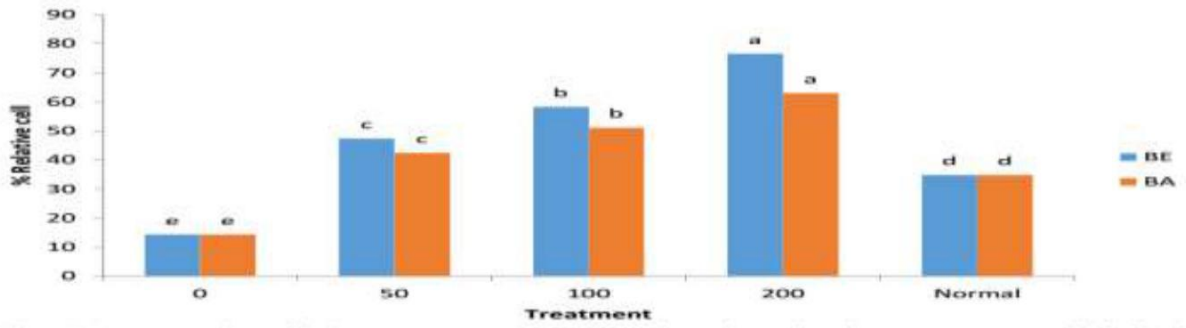



**Figure 2. Improved profile immunocompetent cells by ethanol and water extract of black rice**




**Figur 2. Improved profile immunocompetent cells by ethanol and water extract of black rice**

The relative number of cells undergoing proliferation in the control without extract amounted to 14.40% and amounted to 34.88% of health controls. The higher concentration of the extract, it causes the higher relative number of immune competent cells which proliferate. Ethanol extract and water at doses of 50, 100 and 200 µg/ml respectively was 47.37%, 58.31%, 76.68% and 42.40%, 51.16%, 63.07%.

The relative number of immune competent cells which highest proliferate for the treatment of the ethanol extract of 200 µg/ml is equal to 76.68% and the lowest in the treatment of the water extract was 50 µg/ml is equal to 42.40%. The ethanol treatment extract of black rice and water shows the relative number of proliferating cells significantly higher than controls ($p \leq 0.05$).The ability of ethanol and water extract of black rice in inducing cell proliferation because it contains polyphenols which is quite high and proved have antioxidant activity, thus proved have an imunomodulasi effect [35-36]. In the other research reported that the immune modulating abilities by stimulating proliferation of splenocytes were positively correlated with the content of polyphenols and flavonoids [37].

## Acknowledgment

The authors would like to thank Indonesia Higher Education that has fully funded this research.

## References


1. Decker JM(2000) Introduction to immunology 11 th Hour, Blackwell Science. Inc.
2. Alamgir M, Uddin SJ (2010) Recent advances on the ethnomedicinal plants as immunomodulatory agents, Journal Ethnomedicine, A Source of Complimentary Therapeutics, 227-44.
3. Bellanti JA (1993) *Immunologi III*, Washington: Georgetown University School of Medicine, D.C.
4. Awika JM, Rooney LW (2004) Sorghum Photochemical and Their Potential Impact on Human Health, J. Science Photochemistry, 65(9); 1199-1221.
5. Vinardell MP, Mitjans, M (2008) Immunomodulatory effect of polyphenols, Electronic Journal of Environtment, Agricultural and Food Chemistry, 33-57.
6. Flavonoid imunomodulator, Diambildari: http;// www.depkes.go.id/ index.php? Option = articles& task=view articles & artid=391&itemid=., Diakses 10 February 2017.
7. Abcam, Ab113853 - CFSE Fluorescent Cell Labeling Kit (2013) UK, EU and Row, US. Canada and Latin America, China and Asia Pacific, Japan.
8. Caro GP, Watanabe S, Crozier A, Fujimura T, Yokota T (2013) Photochemical Profile of a Japanese Black-purple Rice, Food Chemistry, 141(7);2821-2827.
9. Chen XQ, Nagao TN, Itani K, Irifune (2012) Anti-oxidative Analysis, and Identification and Quantification of Anthocyanin Pigments in Different Coloured Rice, Food Chemistry, 135(6);2783-2788.
10. Xia X, Ling W, Ma J, Xia M, M Hou, Wang Q (2006) An Anthocyanin-rich Extract from Black Rice Enhances Atherosclerotic Plaque Stabilization in A polipoprotein E Deficient Mice, The Journal of Nutrition, 136 (6); 2220-2225.
11. Wang Q, Xia M, Liu C, Guo H, Hu Q, Zhang Y, Hou M, Zhu H, Ma J, Ling W (2008) Cyanidin-3-O-ß-glucoside Inhibits I NOS and COX-2 Expression by Inducing Liver X Receptor Alpha Activation in THP-1 Macrophages, Life Science, 83 (9);176-184.
12. Zawistowski J, Kopec A, Kitts DD (2009) Effect of a Black Rice Extract (*Oryza sativa* L.) on Cholesterol Levels and Plasma Lipid Parameters in Wistar Kyoto Rats. Journal of Functional Foods, I (7): 50-56
13. Yang Y, Andrews MC, Hu Y, Wang D, Qin Y, Zhu Y (2011) Anthocyanin Extract from Black Rice Significantly Ameliorates Platelet hyperactivity and Hypertriglyceridemia in Dyslipidemic Rats Induced by High Fat Diets. Journal of Agricultural and Food Chemistry, 59 (5); 6759-6764.
14. Lu Y, Foo LY (2008) Polyphenolic Constituents of Black Rice Seed Residue. Food Chemistry, 80 (5): 71-76.
15. Min S, Ryu S, Kim D (2010) Anti-inflammatory Effect of Black Rice, Cyanidin-3-O-ß-glycoside, and Its Metabolites, Cyanidin and Protocatechuic Acid. International Immuno pharmacology, 10(7): 959-966.
16. Nontasan S, Moongngarm A, Deeseenthum S (2012) Application Of Functional Colorant Prepared from Black Rice Bran in Yogurt. Asia-Pacific Chemical, Biological & Environmental Engineering Society, 2(6); 62-67.
17. Hou Z, Qin P, Zhang Y, Cui S, Ren G (2013) Identification of Anthocyanins Isolated from Black Rice (*Oryza sativa* L.) and their degradation Kinetics. Food Research International, 50(8); 691-697.
18. Harborne JB, Metode Fitokimia (1987) Penuntun Cara Modern Menganalisa Tumbuhan, Terbitankedua, Penerbit ITB. Bandung; Hal. 1-8.
19. Shinta Serafinah, I dan, Endang A (2014) Morphological Variation of Six Pigmented Rice Local Varieties Grown in Organic Rice Field in Sengguruh Village, Kepanjen District, Malang Regency. The Journal of Tropical Life Science, 4(2); 149-150.
20. Taie HAA, El-Mergawi R, Radwan S (2008) Isoflavonoids, flavonoids, phenolic acids profiles and antioxidant activity of soybean seeds as affected by organic and bioorganic fertilization. American-Eurasian Journal of Agriculture and Environmental Science, 4(2); 207-213.
21. Rezaeizadeh A, Zuki ABC, Abdollahi M, Goh YM, Noordin MM, Hamid M, Azmi TI (2011) Determination of Antioxidant Activity in Methanolic and Chloroformic extract of Momordica Charantia, Academic Journal, ,4932-4950
22. Syahwar Durre, Raza, Muhammad A (2012) Antioxidant potential of phenolic extracts of Mimusopselengi. Asian Pac J Trop Biomed., 2(7); 547–550.
23. Thomas E (2011) Standard operating procedure for CFSE Proliferation assay, FH-HVTN-A0007, FHCRC HVTN Laboratory.

24. Manian R, Anusuya N, Siddhuraju P, Manian S (2008) The antioxidant activity and free radical scavenging potential of two different solvent extracts of Camellia sinensis (L.) O. Kuntz, Ficusbengalensis L. and Ficusracemosa L. Food Chem. 107; 1000–1007.

25. Koskenkorva-Frank TS, Weiss G, Koppenol WH (2013) The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: Insights into the potential of various iron therapies to induce oxidative and nitrosative stress. Free Radic Biol Med, 65; 1174–94.

26. Hakkim FL, Shankar CG, Girija S (2007) Chemical composition and antioxidant property of holy basil (Ocimum sanctum L.) leaves, stems and inflorescence and there in vitro callus cultures, J Agr Food Chem. 55:9109–9117.

27. Naznin A, Hasan N (2009) In vitro antioxidant activity of methanolic leaves and flowers extracts of lippia Alba, Research Journal of Medicine and Medical Sciences, 4(1); 107-110.

28. Singh PP, Chandra A, Mahdi F, Roy A, Sharma P (2010) Reconvene and reconnect the antioxidant hypothesis in human health and disease. Indian J Clin Bio chem. 25:225–43.

29. Devasagayam TPA, Tilak JC, Boloor KK, Sane Ketaki, S Ghaskadbi, Saroj S, Lele RD (2004) Free Radical and Antioxidants in Human health: Current Status and Future Prospects, JAPI, 52;794-804

30. Nijveldt R, Nood EV, Hoorn DECV, Boelens PG, Norren KV, Leeuven PAMV (2001) Flavonoids: A Review of Probable Mechanisms of Action and Potential Application, Am J Clin Nutr, 74; 418-25.

31. Yen GC, Duh PD, Tsai CL (1993) Relationship between antioxidant activity and maturity of peanut hulls. J Agric Food Chem. 41:67–70.

32. Siddhuraju, P, Mohan PS, Becker K (2002) Studies on the antioxidant activity of Indian laburnum (Cassia fistula L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. Food Chem. 79:61–67.

33. Zhu YZ, Huang SH, Tan BK, Sun J, Whiteman M, Zhu YC (2004) Antioxidants in Chinese herbal medicines: a biochemical perspective. Nat Prod Rep, 21; 478-489.

34. Li HB, Wong CC, Cheng KW, Chen F (2008) Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. Food Sci Techno-LEB, 41; 385-390.

35. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C (2006) The flavonoid quercet in inhibits pro inflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF- κß system. Clin Vaccine Immunology, 13(3); 319-328.

36. Sternberg Z, Chadha K, Lieberman A, Drake A, Hojnacki D, Weinstock-Guttman B, Munschauer F (2009) Immunomodulatory responses of peripheral blood mononuclear cells from multiple sclerosis patients upon in vitro incubation with the flavonoid luteolin: additive effects of IFN-ß. J. Neuron inflammation, 6;28.

37. Lin J, Tang C (2007) Determination of total phenol and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 101; 140-147.