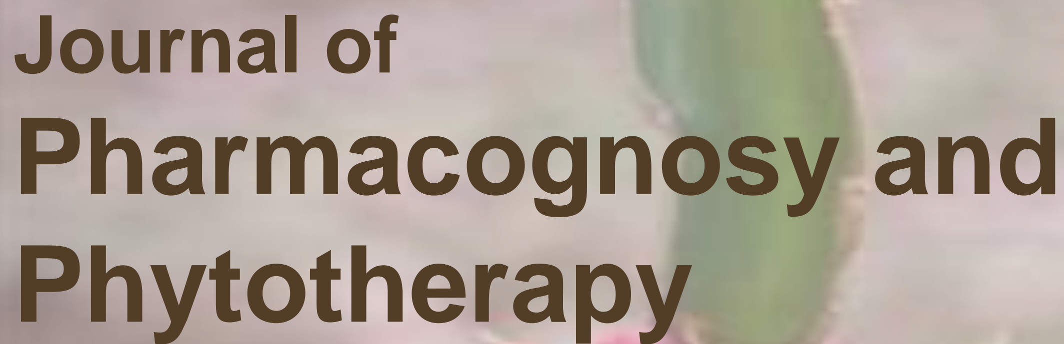

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Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

Amaranthus viridis modulates anti-hyperglycemic pathways in hemi-diaphragm and improves glycogenesis liver function in rats

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Amaranthus viridis **is an ecumenical species in the botanical family of Amaranthaceae, which has been traditionally used to treat several skin diseases along with some antilipidemic activities. The present study was carried out to investigate the anti-hyperglycemic effect of 75% ethanolic extract of *A. viridis* in Neonatal streptozotocin (N-STZ) induced rats' hemi-diaphragm, including screening for secondary plant metabolites. Qualitative phytochemical studies were done by various conventional methods for the possible secondary metabolites. For antidiabetic assay via hemi-diaphragm, Long-Evan rats were used in the study. Type 2 diabetes was induced by a single *ip* injection of streptozotocin to 48 h old pups (N-STZ) and after 3 months, rats were confirmed by an oral glucose tolerance test and further selected for the experiment. Studies to evaluate the glucose utilization capacity of *A. viridis* in isolated rat hemi-diaphragm were done. The data were analyzed by appropriate statistical analysis. *In vitro* glucose uptake by hemi-diaphragm study showed glucose uptake increased significantly in left diaphragm of type 2 diabetes mellitus with insulin alone treated and *A. viridis* alone treated group, where *A. viridis* alone treated group showed very highly significance (p=0.000). Treatment with both insulin and *A. viridis* increased the glucose uptake also very significantly (p=0.004). *A. viridis* extract acted more significantly compared to insulin in T2DM rats. In the normal rats at left hemi diaphragm, *A. viridis* extract also increased glucose uptake more significantly (p=0.009) compared to insulin (p=0.013). At the right diaphragm, glucose uptake increased in all treated groups compared to control group but not significantly. This plant may contain potential anti-hyperglycemic agents which possibly act through some extra pancreatic mechanism that include glucose uptake by diaphragm and increased glycogenesis by liver.**

Key words: *Amaranthus viridis,* antidiabetic, hemi-diaphragm, streptozotocin, glucose, Long-Evan rats.

INTRODUCTION

It is staggering to consider the threat that diabetes poses to our current healthcare system. Recent technological and therapeutical advancement in the management of diabetes mellitus includes pancreas regeneration, islet

transplantation, pancreas transplantation, glucose monitoring at continuous basis, uninterrupted subcutaneous insulin infusion and assorted medication (George, 2009). For mortals with T2DM mellitus (T2DM), an assortment of treatments is available. Most of the pharmacological aid schemes for T2DM are typically grounded on efficacy. Hence, prosperous responses to such therapeutics are frequently variable and unmanageable to predict. In this circumstances, delineation of drug reaction is expected to considerably heighten our ability to provide patients with the most effective treatment strategy given their individual backgrounds. Hence pharmacogenetic analysis of medications against diabetes is still in its early stage. Up to date, major pharmacogenetic acquisitions have focused on biguanides, TZDs and sulfonylureas (Distefano et al., 2010). Most recently researchers have focused on the management diabetes and its associated complications. A variety of approaches have been taken for this purpose.

The plant is usually known as green amaranth or slender amaranth. Possible origin is South America, although widely distributed in tropical weed, foreign to hot-temperate regions and distributed in the tropical and subtropical regions of the world. It is an annual herb with erect or ascending habit, growing to 1 m tall. Leaves are light green and the fruit are obviously wrinkled. It has prominent axillary spines and its leaves can have an obvious reddish or purplish tinge (Stanley et al., 1984). *A. viridis* is found to be a very common garden weed. Also it is found in areas such as roadsides, parks, pastures and other disturbed sites, but seldom cropped, often flattened and prostrate, vacant lots, sometimes crevices of sidewalks and edge of asphalt strips, etc. (Stone, 1970), casual in croplands and waste places too (Whistler, 1988).

The *A. viridis* is a good source of vitamins B and C, taken as vegetables (Sayed et al., 2007). Leaves and seeds are also edible. Previous experiments ascertained it to be a superior source of protein (Macharla et al., 2011). Traditionally it is used to cure eczema, psoriasis and rashes including antinociceptive and antipyretic properties, reported by Kumar (Kumar et al., 2009). Besides these, it is reported by Krishnamurthy that *A. viridis* has anti-inflammatory, antihyperglycemic, hypolipidemic activity as well as acne and skin cleansing property (Krishnamurthy et al., 2011). It has a wide application over diuresis, for snake bites, scorpion stings, dysentery, constipation, eczema, bronchitis, anemia, leprosy and stomach problems like many incidences (Pandhare et al., 2012; Macharla et al., 2011). According to Syed et al. it is quite beneficial to pregnant women to subside labor pains and diabetes (Syed et al., 2007). Its pharmacological study also reveals that it is antiviral (Obi et al., 2006). Meanwhile, its anti-allergenicity is claimed by Sayed et al. (2007). According to Kumar, *A. viridis* is a potent hepatoprotective and antioxidant plant (Kumar et al., 2011). Studies also claimed that it is a good source of anthelmintic and isoproterenol-induced cardiac toxicity inhibitory plant (Ashok et al., 2011; Kumar et al., 2012).

Plants that exhibit activity against hyperglycemia are mainly owing to their ability to bushel the function of pancreatic tissues by causing an alleviation in insulin output or conquer the intestinal assimilation of glucose or aid of metabolites in insulin subordinate processes. Most plants contain cartenoids, terpenoids, glycosides, flavonoids, alkaloids etc. that are usually entailed as having antidiabetic effect (Jung et al., 2006). Type 2 diabetes represents a progressing decline in beta-cell function. Regarding the restrictions of being therapies in fixing the quality of life to normal as well as reducing the risk of chronic diabetic complications by maintaining normal blood glucose level, the search for alternating sources of oral hypoglycemic agents is a requirement. Due to the limitation of recent therapies to control all the metabolic defects of diabetes as well as their possible pathological outcomes with the great expense, there is a clear need for the development of alternative strategies for diabetes treatment.

There has been a possibility of anti-hyperglycemic potentialities of *A. viridis* reported by Krishnamurthy et al. (2011). However, so far hemi-diaphragm pathways of *A. viridis* on Long Evan rats against the hypoglycemic activities, has not been done. So in this study, an attempt was made to evaluate the anti-hyperglycemic activity of ethanolic extract of *A. viridis* plant and also to find out the chemical factors present therein causative for the biological activity. The pharmacological study was carried out on streptozotocin induced type 2 Neonatal model in Long Evan rats. Glucose utilization capacity of bioactivity guided fractions of *A. viridis* in isolated rat hemi-diaphragm in both normal and N-STZ rats was performed and the observed activity was identified, characterized and quantified.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol (PubChem CID: 702), Ferric chloride (PubChem CID: 24380), potassium ferrocyanide (PubChem CID: 11963580), Chloroform (PubChem CID: 6212), sulphuric acid (PubChem CID: 1118), sodium dihydrogen phosphate (PubChem CID: 23672064) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). 4,6-Ethylidene glucose streptozotocin (PubChem CID: 3081692) were purchased from Merck (Darmstadt, Germany) and Human insulin (PubChem CID: 16131099) from Sanofi Bangladesh (Bangladesh). All other chemicals used were from the laboratory stock of the Department of Pharmacology, Bangladesh University of Health Sciences, Dhaka, Bangladesh and were of the highest grade

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available.

Plant mate rial collection and identification

In this study, whole plant part of *A. viridis* (family: amaranthaceae, local name: Notey shak) was used. The plants were collected from Pabna, Bangladesh (Geographic coordinates: Latitude: 24°00′23″ N, Longitude: 89°14′13″ E and elevation above sea level: 19 m = 62 ft) availablesources from the field in the month of June, 2015. The plant was identified by the Bangladesh National Herbarium, Dhaka (Accession No: DACB-38568).

Preparation of ethanol extracts of *Amaranthus viridis*

Mature and fresh whole plants were washed thoroughly after collection and air dried. The weights of plants before and after dry were recorded. After then, the whole plants were grinded and again weighed. Finally, these grind portions were extracted by using 75% ethanolic solvent. The ethanolic extract was prepared by using Soxhlet (Beijing Getty glassware Co. Ltd. maintained at 70°C) and following the completion of extraction was concentrated by using water evaporator (Fujian Snowman Co. Ltd. 5 Litters, asserted at 80°C). The ethanolic extracts at semi dried state were encouraged to dry in a freeze drier (HETOSICC, Heto Lab Equipment, Denmark) at -55°C temperature and preserved in a reagent bottle at -8°C in a freezer for analysis.

Preparation of animals for treatment

Adult Long Evans rats weighting 160 to 220 g were included in the study. The animals were bred at Bangladesh Institute of Research and Rehabilitation for Diabetes, Endocrine and Metabolic Disorders (BIRDEM) animal house, Dhaka, Bangladesh, maintained at a constant room temperature of 22±5°C with humidity of 40 to 70% and the natural 12 h day-night cycle. Animal housing and handling were performed in accordance with Good Laboratory Practice (GLP) mentioned in US guidelines (NIH publication # 85-23, revised in 1985). The experimental protocols were critiqued and sanctioned by the Institutional Animal Ethics Committee prior to initiation of the experiment. The rats were fed upon a stock lab pellet diet and water supplied *ad libitum*. Standard rat diet contained wheat (40%), wheat bran (20%), fish meal (10%), germ (3.9%), oil cake (10%), milk (3.8%), pulses (3.9%), soyabean oil (1.5%), rice polishing (5%), molasses (0.95%) and salt (0.95%). Embavit GS (vitamin mixture) 250 g was added per 100 kg of rat food. The influence of circadian rhythm was avoided by starting all experiments at 7:30 am. The experiments were conducted according to the ethical guidelines approved by Bangladesh Association for Laboratory Animal Science.

Preparation of type 2 diabetes model rats

Type 2 diabetes was hastened by a single *ip* injection of streptozotocin (STZ) dissolved in citrate buffer (10 ml), at a dose of 90 mg/kg of body weight into the rat pups (48 h old, average weight 7 g) as described by Weir and Bonner-Weir et al. (2013). Following 3 months of STZ injection, rats were examined by oral glucose tolerance test (OGTT) for their blood glucose level. Diabetic model rats with blood glucose level >7.00 mmol/L, at fasting condition were selected for studying the effects of *A. viridis* extracts.

Phytochemical screening of *A. viridis*

Three (3) g of *A. viridis* 75% ethanolic extract was boiled with 30 ml distilled water for 5 min in a water bath and was filtered while hot. The extract sample or filtrate was taken for the experiments wherever applicable using standard protocols (Sharmistha et al., 2012) to test the presence of bioactive compounds.

In vitro glucose uptake study of *A. viridis* by isolated rat hemi-diaphragm

Glucose uptake by rat hemi-diaphragm was estimated by the methods described elsewhere (Walaas and Walaas, 1952; Chattopadhyay et al., 1992) with some modifications. 32 male and female Long Evans normal and Type 2 rats were weighed and rats weighing between 170 to 210 g were used in the study. The weight of the rats were measured before and after fasting. Four sets containing graduated test tubes (n=4) for each hemi-diaphragm were taken. Group I served as a control which contained 2 ml of Tyrode (NaCl (8 gm/L), KCl (0.20 gm/L), $CaCl_2$ (0.20 gm/L), $MgCl_2$ (0.10 gm/L), NaH_2PO_4 (0.05 gm/L), $NaHCO_3$ (1 gm/L), Glucose (1 gm/L) having pH 6.5) solution with 2% glucose, Group II contained 2 ml Tyrode solution with 2% glucose and regular insulin (Novo Nordisk) 0.62 ml of 0.4 units per ml solution. Group III contained 2 ml Tyrode solution with 2% glucose and 1.38 ml of *A. viridis* extract (30 mg extract dissolved in 3 ml H_2O and adjust PH to 7.4) and the Group IV contained 2 ml Tyrode solution with 2% glucose and regular insulin 0.62 ml of 0.4 units per ml solution and 1.38 ml of *A. viridis* extract. The volumes of all the test tubes were made up to 4 ml with distilled water to match the volume of the test tubes of Group IV. Long Evans rats were tested overnight and killed by decapitation. The diaphragms were dissected out quickly with minimal trauma and divided into two halves. Two diaphragms from the same animal were not used for the same set of experiment. Four numbers of diaphragms were used for each group. The hemi-diaphragms were placed in test tubes and incubated for 30 min at 37°C in an atmosphere of 100% oxygen with shaking at 140 cycles/min. Glucose uptake per gram of tissue was calculated as the difference between the initial and final glucose content in the incubated medium.

Estimation of glucose level

The glucose oxidase (GOD-PAP) test was performed using the established method published by Trinder (Trinder, 1969) without modification.

Statistical analysis

Data from the experiments were analyzed using the Statistical Package for Social Science (SPSS) software for windows version 21 (SPSS Inc., Chicago, Illinois, USA). All the data were expressed as Mean ± SD or as Median (Range) as appropriate. Statistical analysis of the results was performed by using the student's t-test (paired and unpaired), ANOVA (analysis of variance) followed by Bonferroni and Dunnett post hoc test and Mann Whitney (u) test. GraphPad Prism (Version 5) software was used for all statistical analysis and P<0.05 was considered as significance.

RESULTS

Phytochemical screening of 75% ethanolic extract of *A. viridis*

The present investigation was carried out to assess the

Table 1. Phytochemical screening for the possible secondary metabolites of the *A. viridis* extract.

Serial no	Phytochemicals	Results
1	Tannin	++
2	Saponin	++
3	Alkaloid	++
4	Flavonoid	++
5	Phenol	++
6	Steroid	-
7	Terpenoid	+
8	Carbohydrate	-

++ = Presence; + = trace; - = absence.

qualitative phytochemical analysis of 75% ethanolic extract of *A. viridis.* The phytochemical screening reveals the presence of various plants' secondary metabolites as shown in Table 1. Tannin, saponin, alkaloid, flavonoid and phenols were found in the extract of *A. viridis.* Terpenoid was in trace amount but carbohydrate and steroids were not found in our phytochemical studies.

Check values of STZ induced Type 2 diabetic rats in different groups

To generate a rat model mimicking human type 2 diabetes with impaired insulin secretion and insulin resistance, we used STZ injection (90 µg/kg, body weight) to 48 h old pulps. STZ injection to neonates led to the injury of the pancreas resulting in destruction of the functional β-cells. At the age of 3 months, when an oral glucose challenge (500 mg/kg, body weight) was done, the remaining β cell could not cope with the load, which reflected in the postprandial rise of serum glucose level at 30 min (Table 2). The rise was significant among all rats compared to baseline value. On the basis of this experiment these rats were selected and sorted into three distinct groups to carry out the experiments with feeding of different extracts.

Effect of *A viridis* extracts using glucose uptake by isolated rat-hemi (left) diaphragm on normal rats in vitro assay

In order to explore the mechanism underlying the anti-hyperglycemic activity of *A. viridis in vitro* glucose uptake study of rat hemi-diaphragm was done. The results of glucose uptake by the left hemi-diaphragm in normal rats are presented in Table 3 and Figure 1. The results showed that glucose uptake was enhanced by hemi-diaphragm when the normal rats were treated with insulin alone (glucose uptake m ± SD, mg/g/30 min, control 2.02 ± 0.62 vs insulin 6.59 ± 2.01; p=0.013). When hemi-diaphragm was treated with *A. viridis* extract, glucose uptake was also increased significantly (glucose uptake m ± SD, mg/g/30 min, control 2.02 ± 0.62 vs plant extract 6.93 ± 0.62; p = 0.009). When the hemi-diaphragm of normal rats was exposed to both insulin and *A. viridis* extract, it did not increase glucose uptake significantly.

Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (right) diaphragm on normal rats *in vitro* assay

The results of glucose uptake by rat right hemi-diaphragm in normal rats is presented in Table 4 and Figure 2. The results showed that glucose uptake was enhanced by hemi-diaphragm when the normal rats were treated with insulin alone (glucose uptake m ± SD, mg/g/30 min, control 0.568 ± 0.350 vs insulin 2.684 ± 2.527; p = 0.148). When hemi-diaphragm was treated with *A. viridis* extract, glucose uptake was also increased but not-significantly (glucose uptake m ± SD, mg/g/30 min, control 0.568 ± 0.350 vs plant extract 1.893 ± 1.704; p = 0.178). When the hemi-diaphragm of normal rats was exposed to both insulin and *A. viridis* extract, it also did not increase glucose uptake significantly.

Effect of *A viridis* extracts using glucose uptake by isolated rat-hemi (left) diaphragm on STZ induced type 2 rats *in vitro* assay

The results of glucose uptake by rat left hemi-diaphragm in T2DM rats is presented in Table 5 and Figure 3. When the T2DM rats were treated with insulin alone the glucose uptake was significantly increased in compared to control (glucose uptake m ± SD, mg/g/30 min, control 2.55 ± 0.36 vs insulin 5.26 ± 1.21; p = 0.037). In the treatment with *A. viridis* extract, glucose uptake was significantly higher in comparison to control (glucose uptake m ± SD, mg/g/30 min, control 2.55 ± 0.36 vs plant extract 9.74 ± 0.87; p = 0.000) as well as insulin alone (glucose uptake m ± SD, mg/g/30 min, insulin 5.26 ± 1.21 vs plant extract 9.74 ± 0.87; p = 0.002), respectively.

Insulin with *A. viridis* extract was also exposed to a significant increased glucose uptake when it compared with control (glucose uptake m ± SD, mg/g/30 min, control 2.55 ± 0.36 vs insulin with plant extract 6.46 ± 0.93; p = 0.004) as well as plant extract alone (glucose uptake m ± SD, mg/g/30 min, plant extract 9.74 ± 0.87 vs insulin with plant extract 6.46 ± 0.93; p = 0.012), respectively.

Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (right) diaphragm on STZ induced type 2 rats *in vitro* assay

The results of glucose uptake by rat right hemi-diaphragm in T2DM rats is presented in Table 6 and

Table 2. Check values of STZ induced Type 2 diabetic rats in different groups.

Groups	Fasting (0 min)	After OGTT (30 min)
WC (n=6)	7.44±0.57 (100%)	14.62±1.92 (197%)
Glc (n=6)	7.71±0.55 (100%)	14.82±1.35 (192%)
AVEtE (n=6)	7.72±0.87 (100%)	14.73±1.39 (191%)

Paired samples T test	
Groups	**0 min vs 30 min**
WC (n=6)	0.000
Glc (n=6)	0.000
AVEtE(n=6)	0.000

Results are expressed as Mean ±SD. OGTT= Oral Glucose Tolerance Test, STZ= Streptozotocin; WC = Type 2 Water Control; Glc = Type 2 Glibenclamide treated group and AVEtE= 75% ethanol extract of *A. viridis.*

Table 3. Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (Left) diaphragm on normal rats *in vitro* assay.

Group (G)	Treatment	Incubation medium	Glucose uptake (mg/g/30 min)	p value
G1	Control	Tyrode solution with Glucose (2%)	2.02±0.62 (100%)	-
G2	Insulin	Tyrode solution with Glucose and Insulin (Actrapid 40 u/ml)	6.59±2.01 (326%)	G1 Vs G2, p=0.013*
G3	Plant extract	Tyrode solution with Glucose and plant extract (30 mg/3 ml H_2O)	6.93±0.62 (343%)	G1 Vs G3, p=0.009**
G4	Insulin+ plant extract	Tyrode solution with Glucose, Insulin and plant extract	4.38±1.25 (211%)	G1 Vs G4, p= 0.021*

Results were expressed as Mean ±SD. Statistical analysis between group comparison was done by using one-way ANOVA with post hoc Bonferroni test. *= p<0.05; **=p<0.01.

Table 4. Effect of *A viridis* extracts using glucose uptake by isolated rat-hemi (Right) diaphragm on normal rats *in vitro* assay.

Group (G)	Treatment	Incubation medium	Glucose uptake (mg/g/30 min)	p value
G1	Control	Tyrode solution with Glucose (2%)	0.568±0.350 (100%)	
G2	Insulin	Tyrode solution with Glucose and Insulin (Actrapid 40 u/ml)	2.684±2.527 (471%)	G1 Vs G2, p=0.148
G3	Plant extract	Tyrode solution with Glucose and plant extract (30mg/3ml H_2O)	1.893±1.704 (333%)	G1 Vs G3, p=0.178
G4	Insulin+ plant extract	Tyrode solution with Glucose, Insulin and. plant extract	1.898±0.977 (334%)	G1 Vs G4, P=0.067

Results were expressed as Mean ±SD. Statistical analysis between group comparison was done by using one-way ANOVA with post hoc Bonferroni test. *= p<0.05; **=p<0.01.

Figure 4. When the T2DM rats were treated with insulin alone the glucose uptake was increased by 59% compared to control group but not significantly. In the treatment with *A. viridis* extract, glucose uptake was 84% higher in comparison to control group. Glucose uptake was found to be increased up to 119% in case of

Table 5. Effect of *A.viridis* extracts using glucose uptake by isolated rat-hemi (Left) diaphragm on STZ induced type 2 rats *in vitro* assay.

Group (G)	Treatment	Incubation medium	Glucose uptake (mg/g/30 min)	p value
G1	Control	Tyrode solution with Glucose (2%)	2.55±0.36 (100%)	
G2	Insulin	Tyrode solution with Glucose and Insulin (Actrapid 40 u/ml)	5.26±1.21 (206%)	G1 Vs G2, **p=0.037***
G3	Plant extract	Tyrode solution with Glucose and plant extract (30 mg/3 ml H_2O)	9.74±0.87 (381%)	G1 Vs G3, **p=0.000****, G2 Vs G3; **p=0.002****
G4	Insulin+ plant extract	Tyrode solution with Glucose, Insulin and plant extract	6.46±0.93 (253%)	G1 Vs G4, **p=0.004****, G3 Vs G4, **p=0.012***

Results were expressed as Mean ±SD. Statistical analysis between group comparison was done by using one-way ANOVA with post hoc Bonferroni test. *= p<0.05; **=p<0.01.

Table 6. Effect of *A viridis* extracts using glucose uptake by isolated rat-hemi (right) diaphragm on STZ induced type 2 rats *in vitro* assay.

Group (G)	Treatment	Incubation medium	Glucose uptake (mg/g/30 min)	p value
G1	Control	Tyrode solution with Glucose (2%)	1.385±0.980 (100%)	
G2	Insulin	Tyrode solution with Glucose and Insulin (Actrapid 40 u/ml)	2.195±1.293 (159%)	G1 Vs G2, p=0.359
G3	Plant Extract	Tyrode solution with Glucose and plant extract (30mg/3ml H_2O)	2.548±1.423 (184%)	G1 Vs G3, p=0.233
G4	Insulin+ Plant Extract	Tyrode solution with Glucose, Insulin and. plant extract	3.033±0.823 (219%)	G1 Vs G4, P=0.043*

Results were expressed as Mean ±SD. Statistical analysis between group comparison was done by using one-way ANOVA with post hoc Bonferroni test. *= p<0.05; **=p<0.01.

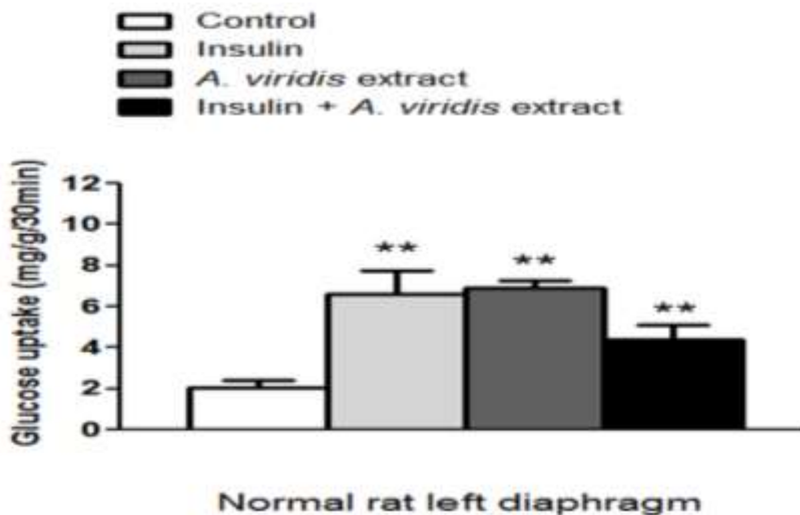

Figure 1. Effect of *A viridis* extracts using glucose uptake by isolated rat-hemi (Left) diaphragm on normal rats *in vitro* assay. *= p<0.05; **=p<0.01.

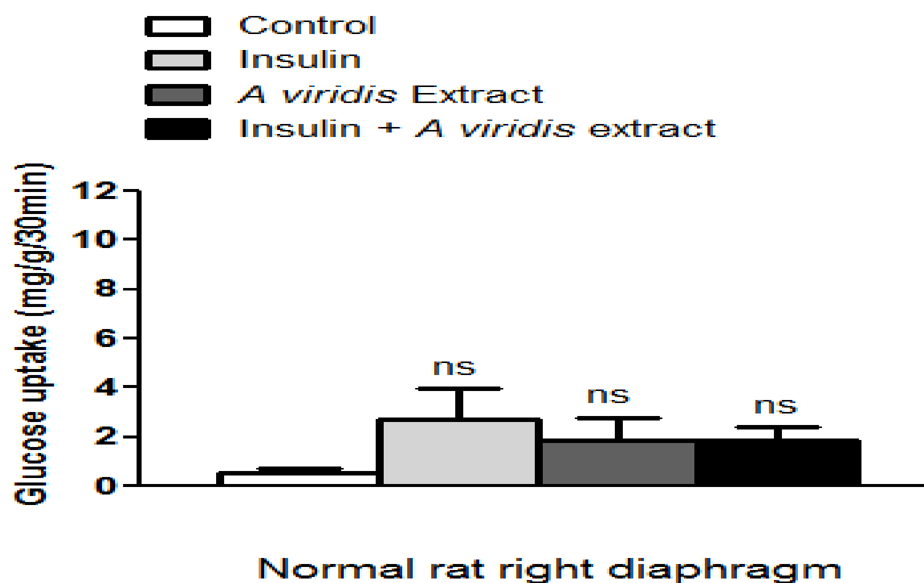

Figure 2. Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (Right) diaphragm on normal rats *in vitro* assay. *= p<0.05; **=p<0.01 and ns= not significance.

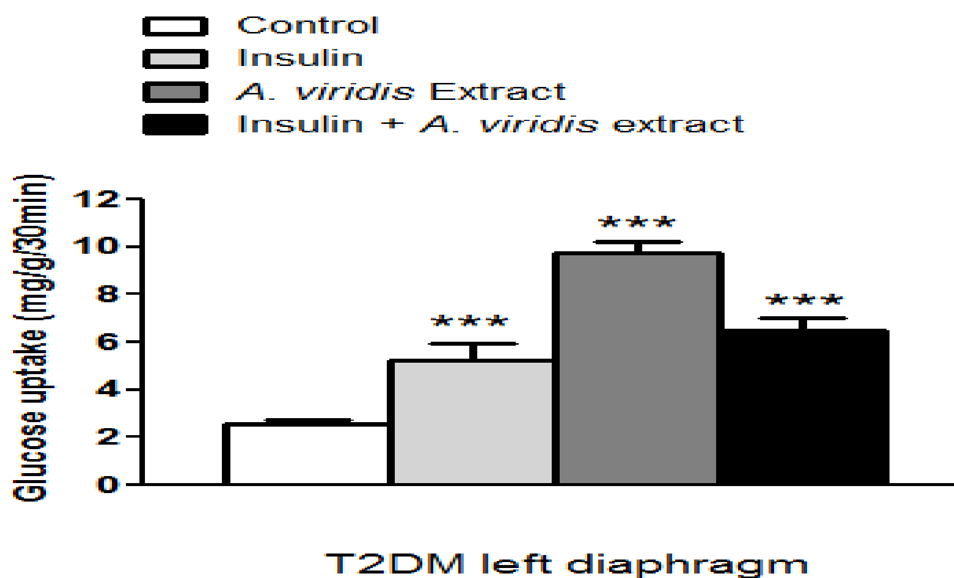

Figure 3. Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (Left) diaphragm on STZ induced type 2 rats *in vitro* assay. *= p<0.05; **=p<0.01.

A. viridis extract with insulin, compared with control group as well as plant extract and insulin individually.

DISCUSSION

Previous studies have shown that antidiabetic plants possess the presence of alkaloids, glycosides and polyphenols like phytoconstituents (Sharmistha et al., 2012).

Therefore in the beginning of the study, preliminary phytochemical screening of the 75% ethanolic extract of whole plant for secondary plant metabolites was performed. The results revealed the presence of saponin, tannin, flavonoids, alkaloids, terpinoids, phenol (Table 1). The presence of a significant number of secondary plant

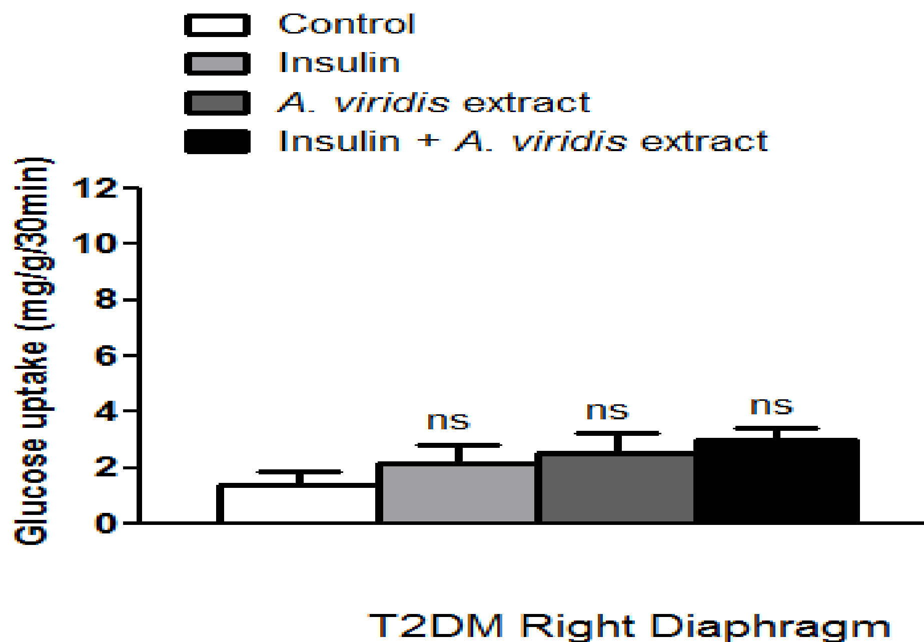

Figure 4. Effect of *A. viridis* extracts using glucose uptake by isolated rat-hemi (right) diaphragm on STZ induced type 2 rats *in vitro* assay. ns = not significance.

metabolites in *A. viridis* might be responsible for the biological activities observed later in this study.

Type 2 diabetes was developed by injecting STZ (a pancreatic β cell toxin) to 48 h old pups, which inside the β-cell dissociates into glucose and methylnitrogenase. The later alkaylates and modified biomolecules breakdown DNA and destroys β cell, thereby causing diabetes. Early injury of the β cells resulted in the partial recovery of β cell leading to type 2 diabetes as a result of insulin resistance in target tissues and impaired insulin secretion, accompanied by increased adiposity.

When at the age of 3 months these rats have been challenged with an oral glucose load, all of them could not cope with the glucose load due to the defective β cells. Although their fasting glucose values were a bit higher (ranging from 7.44 to 7.72), indicating the presence of some functioning β cells but their post challenge glucose values were significantly higher which proved that these rats have developed type 2 diabetes (Table 2).

Therefore, it may be assumed that the hypoglycemic activity of *A. viridis* in type 2 model rats at least, may be partly due to increased uptake of glucose for the formation of glycogen by enhanced glycogenesis. To put further insight regarding the mechanism of anti-hyperglycemic effect of *A viridis* extract in both normal and T2DM rats *in vitro* glucose uptake by hemi-diaphragm was performed.

In this study, individual insulin (p = 0.013) and *A. viridis* (p = 0.009) treated group showed a significant increment in glucose uptake in normal rats at left diagram (Tables 3 and 4). Hence, in case of T2DM rats p = 0.037 with insulin alone and p = 0.000 with *A. viridis* extract (Table 5). Moreover, treatment with both insulin and *A. viridis* extract caused significantly much higher glucose uptake by rat left hemi-diaphragm (p = 0.004). Thus it can be concluded that *A. viridis* improves hyperglycemia by extrapancreatic mechanism as the left diaphragm improves glucose uptake more than right diaphragm.

Conclusion

The present study demonstrates that phytochemical screening of 75% ethanol extract contains a number of secondary plant metabolites including flavonoids, alkaloids which might be associated with the obtained antidiabetic properties of *A. viridis. In vitro* glucose consumption by hemi-diaphragm study exhibited increased state of the glucose by hemi-diaphragm in the presence of *A. viridis* extract. From the findings it can be concluded that different secondary metabolites of plant materials had some extra pancreatic mechanism like glucose consumption by peripheral tissues. Thus, the plant might be considered for further chemical studies and detailed toxicological studies for future drug development.

Conflict of interest

The authors have not declared any conflict of interest.

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Abbreviations

N-STZ, Neonatal streptozotocin; ***A. viridis*****,** *Amaranthus viridis*; **T2DM,** type 2 diabetes mellitus; **BIRDEM,** Bangladesh Institute of Research and Rehabilitation for Diabetes, Endocrine and Metabolic Disorders; **GLP,** good laboratory practice; **OGTT,** oral glucose tolerance test; **GOD-PAP,** glucose oxidase; **SPSS,** statistical package for social science; **ANOVA,** analysis of variance; **M±SD,** mean ± standard deviation.

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Full Length Research Paper

Evaluation of antioxidant and anti-tyrosinase activities as well as stability of green and roasted coffee bean extracts from *Coffea arabica* and *Coffea canephora* grown in Thailand

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Coffea arabica **(Arabica) and** ***Coffea canephora*** **(Robusta) are the economic plants in Thailand that are widely cultivated in Northern and Southern Thailand. This study aims to evaluate the antioxidant, anti-tyrosinase activities, toxicity, stability and identify chemical components of the coffee bean extracts. The best extract that showed good biological activities will be further used to develop cosmeceutical products. Green and roasted coffee beans from two species were extracted with hexane following ethanol by maceration. Their antioxidant activities were detected by 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and lipid peroxidation inhibition assays. In addition, anti-tyrosinase activity was also evaluated. The results revealed that the ethanolic coffee bean extracts showed a higher level of antioxidant activity than in the hexane extracts. All extracts also possessed a considerable anti-tyrosinase activity, but less potent than kojic acid and arbutin. Chemical compounds of these extracts were determined using caffeine and chlorogenic acid as standards of reference by the thin layer chromatography and the high performance liquid chromatography. The green coffee bean extracts consisted of caffeine and chlorogenic acid while the roasted coffee bean extracts presented only caffeine due to a few chlorogenic acid content after the roasting process. The ethanolic coffee bean extracts that showed good activities were selected to be evaluated on toxicity and stability. The selected extracts were kept at various storage conditions to evaluate their stability using DPPH assay and anti-tyrosinase activity assay. The result showed that the extracts were not toxic to cells. Therefore, the extracts were safe to be components in skin care products. After the stability test, the extracts indicated a good stability and activities. These results led to the conclusions that the coffee bean extracts possess a good biological activities and are assumed to be promising natural active ingredients with a good stability profile for further development of cosmeceutical or anti-aging products.**

Key words: *Coffea arabica*, *Coffea canephora,* green coffee bean, roasted coffee bean, antioxidant activity, anti-tyrosinase activity.

INTRODUCTION

Many factors such as environmental conditions, UV radiation, foods, stress as well as pollutants are all causes of free radicals formation in the body. Free radicals can induce many diseases such as different

types of cancer, coronary artery disease, nervous system diseases, lung diseases and also rheumatoid arthritis (Devasagayam et al., 2004; Pham-Huy et al., 2008). Moreover, they play an important role in tissue aging, including skin aging (Farage et al., 2008; Poljsak et al., 2012). It is a never-ending endeavor for researchers in attempt to find the new active ingredients to counteract the aging process, especially the focus on antioxidant or anti-free radical capability and also anti-tyrosinase activity; which are involved in the prevention of skin aging and help to generate skin brightening. Numerous Thai plants have been used as health care and cosmetic products for many decades.

Coffee is one of the economic plants which is widely grown in Thailand. It is a native plant of Africa in Rubiaceae family and it is very popular around the world, especially Southeast Asia (Charrier and Berthaud, 2012). *Coffea arabica* (Arabica) is popularly cropped in the Northern part of Thailand while *Coffea canephora* (Robusta) is mostly cultivated in Southern Thailand. They are different in the seed shape, smell and taste (Chuakul et al., 1997). Robusta coffee is a major production in Thailand, with about 80,000-85,500 tons per year, whereas Arabica coffee production is only approximately 800-850 tons per year. Sixty percent of the Robusta coffee is exported and mostly used for instant coffee production. Most of Arabica coffee is used in roasted and ground coffee for the domestic market.

Previous studies showed that drinking coffee could reduce risk of Parkinson, Alzheimer, hypertension, diabetes type 2 and cancers, and also promote the liver function (Chu et al., 2011; Cano-Marquina et al., 2013; O'Keefe et al., 2013).

In addition, coffee beans serves as antioxidant, anti-inflammatory, for inhibition of albumin denature, UV radiation protection, and in anti-bacterial activities (Antonio et al., 2011; Wagemaker et al., 2011; Almeida et al., 2012; Chandra et al., 2012; Moreira et al., 2013; Liang et al., 2016). Therefore, coffee beans are an interesting option to select for the development of cosmeceutical products in the future. Previous phytochemical studies of coffee indicated that green coffee beans consisted of caffeine, caffeic acid, chlorogenic acid and trigonelline, whereas roasted coffee beans are composed of caffeine, trigonelline, chlorogenic acid, and melanoidin (Liu et al., 2011; Vignoli et al., 2011; Moreira et al., 2013). The chemical components that are mentioned above indicate that coffee beans are a great source for antioxidant.

The data from this research will be used to develop further cosmeceutical products. Therefore, the aims of to select the best extract from antioxidant, lipid peroxidation inhibition and anti-tyrosinase activities. This study are to choose the good solvent extraction and research also shows toxicity of selected coffee bean extracts and stability at various storage conditions. Moreover, the research attempts to identify the chemical constituents of coffee bean extracts by thin layer chromatography and high performance liquid chromatography to confirm active compounds in the extracts.

MATERIALS AND METHODS

Plant materials, chemicals and enzymes

Green and roasted coffee beans (Arabica and Robusta) were obtained from a coffee farm in Chiang Mai province in the northern part of Thailand. The best geography and environment for cultivating coffee include clay soil with high potassium, pH range between 4.5 and 6.5, and rainfall 1,500 and 2,300 ml per year. Arabica coffee is grown with the open-system without shade, the temperature of 15 and 26°C, 80% humidity at 1,000 to 1,700 m above sea level in Chiang Mai, Thailand. Arabica coffee cherries were harvested in October, they were prepared by the pulping process, the wet fermentation process, and the sun drying process. Green coffee beans were then transferred from a high efficiency hulling machine where the final layer of parchment was completely removed. Robusta coffee is grown with the open-system with shade, the temperature of 23 – 32°C, 90% humidity at 700 to 1,000 m above sea level in Chumphon province, Thailand. Robusta coffee cherries were harvested in November. Green coffee beans were prepared the same way as Arabica green coffee beans. Roasted coffee beans were prepared in a high quality, fully automated roaster and sealed in 4-layer-foil bags embedded with one way air valves at 210 - 240°C for 10 to 20 min (medium roast). The green and roasted coffee beans were stored away from light at the room temperature.

Turmeric extract and mangosteen extract were obtained from a cosmetic laboratory at Chiang Mai University, Chiang Mai, Thailand. Caffeine, chlorogenic acid, mushroom tyrosinase and L-tyrosine were purchased from Sigma-Aldrich, USA. L-dopa was purchased from Isotec. Trolox, gallic acid, quercetin, 2,2-diphenyl-1-picryl hydrazyl (DPPH), Folin-Ciocalteu reagent and linoleic acid were purchased from Sigma Chemical Co., (USA). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2, 2' azobis 2-amidinopropane dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Japan. RAW 264.7 cells were purchased from American Type Culture Collection (USA). MTT dye was purchased from Bio Basic (Markham, Canada). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco. Acetonitrile and acetone were purchased from RCI Labscan Ltd., Thailand.

Extractions

Green and roasted coffee beans were grounded into powder before being extracted with hexane by maceration for three days. Then filtered with Whatman No. 1 filter paper and the filtrates were evaporated to concentrated extracts by rotary evaporator. The obtained extracts were named as hexane green Arabica bean extract (HGA), hexane roasted Arabica bean extract (HRA), hexane green Robusta bean extract (HGR) and hexane roasted Robusta

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bean extract (HRR).

After that, each residue after hexane extraction was dried and extracted with 95% ethanol by maceration for three days, filtered and evaporated by rotary evaporator. The obtained extracts in this part were named as ethanolic green Arabica bean extract (EGA), ethanolic roasted Arabica bean extract (ERA), ethanolic green Robusta bean extract (EGR), and ethanolic roasted Robusta bean extract (ERR). All the extracts were kept in light resistant well-closed container in a freezer of a refrigerator for further investigations.

Determination of total phenolic content

The coffee bean extracts were determined for total phenolic content by Folin-Ciocalteu assay (Johnson et al., 2008; Garzón et al., 2009). Each sample was dissolved in ethanol (1 mg/ml) and then the 500 µl was transferred into a test tube, mixed with Folin-Ciocalteu reagent then Na_2CO_3 7.5% w/v was added. The mixtures were mixed with a vortex mixer and incubated for 30 min in the dark. The absorbance was measured at 765 nm using a spectrophotometer (Shimadzu UV-Vis 2450, Japan). The concentration of total phenolic content in all extracts was calculated as gallic acid equivalent (GAE), in milligram gallic acid/gram of a dry sample.

Determination of antioxidant activities

DPPH radical scavenging assay

The stable free radical DPPH ($DPPH^{\bullet}$) reacted with antioxidants and produced colorless 2,2-diphenyl-l-picryl hydrazine. The more colorless sample indicated the high antioxidant activity. Different concentrations of extracts were dissolved in ethanol and tested with freshly prepared 180 µl of DPPH• in ethanol. The mixtures were then mixed with a vortex mixer and incubated in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 520 nm with a microplate reader (DTX 880 multimode detector) (Brem et al., 2004). The percentage of inhibition was calculated by the equation:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where, $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the test sample. The half maximal inhibitory concentration (IC_{50}) was calculated from the curve between the percentage of inhibition and the concentration of extract. Gallic acid, trolox and quercetin were used as standard antioxidants.

ABTS cation radical scavenging assay

ABTS stock solution was prepared by mixing 7 mM ABTS with 140 mM $K_2S_2O_8$ and kept in the dark at room temperature for 16 h before use (Tang et al., 2004). The ABTS stock solution was diluted with deionized water to obtain the absorbance of 0.9±0.1 at 734 nm. The extracts were dissolved in ethanol and then 10 µl of each sample was mixed with 1 ml of ABTS solution. The mixture was kept for 6 min and was then measured for the absorbance at 734 nm using the spectrophotometer.

The absorbance was used to calculate percentage inhibition of antioxidant and IC_{50} value when compared with gallic acid, trolox and quercetin.

Lipid peroxidation inhibition (linoleic acid) assay

The extracts were diluted with ethanol before used. Each sample (200 µl) was mixed with 800 µl of phosphate buffer (pH 7.0), 200 µl of ethanol, 400 µl of deionized water, 400 µl of 2.5% linoleic acid and 80 µl of AAPH in a test tube. The mixture was incubated in the dark at 37°C for 24 h to generate the lipid peroxidation. After that, the mixture was tested by the ferric thiocyanate method. The mixture reacted with $FeCl_2$ and ammonium thiocyanate for 5 min. The absorbance was measured at 500 nm using a spectrophotometer.

The absorbance was used to calculate the percentage in the inhibition of lipid peroxidation activity and IC_{50} value when compared with gallic acid, trolox and quercetin.

Determination of mushroom tyrosinase inhibition activity

Each extract was dissolved in ethanol at the concentration of 2.5, 100 µl of each sample was added to the 96-well plate and then 40 µl of 2.5 mM L-dopa or 2.5 mM L-tyrosine solution were added to the well plate, then incubated at 37°C for 5 min before adding 60 µl of mushroom tyrosinase enzyme (Pomerantz et al., 1963). The mixture was incubated again at 37°C for 15 min before determining the absorbance at 450 nm with the microplate reader. Kojic acid, ellagic acid, α-arbutin and β-arbutin were used as reference tyrosinase inhibitors. The percentage inhibition of tyrosinase activity was calculated as followed:

$$\text{inhibition (\%)} = [(A_a - A_b)/ A_a] \times 100$$

Where A_a = absorbance without a test sample and A_b = absorbance with a test sample.

Cell culture and MTT assay

The cell culture was adapted from the previous study of Mueller et al. (2010). Briefly, RAW 264.7 cells were seeded at a density of 2×10^6 cells per well in 24 well plates, and incubated for 24 h at 37°C. On the following day, the extracts in ethanolic solution were added, and cells were incubated for a further 24 h at 37°C. Then, the media was removed and MTT was added to the cells, and the cells were incubated for 2 h at 37°C. The supernatant was then removed, and the cells were lysed with lysis buffer (10% SDS in 0.01 N HCl). The optical density at 570 nm, corrected by the reference wavelength 690 nm, was measured using a microplate reader.

Determination of TLC chromatogram

The extracts with good antioxidant and anti-tyrosinase activities were selected for TLC analysis. Caffeine and chlorogenic acid were used as standards. The extracts were performed for TLC fingerprints on Merck Silica gel 60 F254 plates. The solvent system was toluene : ethyl acetate : water : formic acid (15:90:5:5) (Adham, 2015). Then, the chromatogram was detected under short wavelength UV (246 nm) and R_f values were calculated when compared with caffeine and chlorogenic acid. The R_f values were calculated from the equation:

$$R_f = \text{distance traveled by substances/distance traveled by solvent}$$

Identification of chemical components of extracts using HPLC

Chlorogenic acid and caffeine were determined using HPLC model 1100 (Agilent®, USA). All samples were filtered with 0.45 µm filter paper. Ten microliters of samples was injected into a C18 column (Mightysil®, Japan). The mobile phase consisted of acetonitrile and

Table 1. Percentage yield of coffee bean extracts.

Extracts		Yield (%)	Physical appearances	pH
Hexane	Green Arabica (HGA)	4.82	Yellow color extract	5
	Roasted Arabica (HRA)	11.37	Brown color extract	5
	Green Robusta (HGR)	1.93	Yellow color extract	5
	Roasted Robusta (HRR)	12.07	Brown color extract	5
Ethanol	Green Arabica (EGA)	1.93	Yellow color extract	5
	Roasted Arabica (ERA)	6.51	Dark brown color extract	5
	Green Robusta (EGR)	2.17	Green color extract	5
	Roasted Robusta (ERR)	5.43	Dark brown color extract	5

1% acetic acid (pH 3) with ratio of 15:85 at a flow rate of 1.0 ml/min (Ayelign et al., 2013). Chromatograms were recorded at 280 nm. Identification of chlorogenic acid and caffeine in extracts was performed by comparing the retention time and chromatogram with their reference standard compounds.

The stability of coffee bean extracts

The extracts with good biological activities were selected for a stability study in which the extracts were kept at various storage conditions: room temperature (RT), room temperature in the dark (DRT), 4 and 45°C for 3 months. In addition, they were kept in accelerated conditions: heating-cooling cycling: 45°C for 48 h and then moved to 4°C for 48 h (1 cycle) for 6 cycles. After each condition, the extracts were analyzed on their antioxidant activity by DPPH assay and anti-tyrosinase activity.

Statistical analysis

All the experiments were done in triplicate and data were showed as mean ± standard deviation (sd). One-way analysis of variance (ANOVA) was carried out to determine the significant difference of the data between the green and roasted coffee bean extracts and standards at the level of p-value < 0.05 using software SPSS (Version 19.0, IBM).

RESULTS AND DISCUSSION

The yield of extracts

The coffee bean extracts obtained from hexane and ethanol maceration were calculated with percentage yield which ranged between 1.93 and 12.07% as shown in Table 1. The results showed that HGA, HGR and EGA were semisolid with a yellow color and unique odor. HRA, HRR, ERA and ERR were semisolid with brown or dark brown color and coffee odor whereas the EGR was green semisolid with a unique odor. All the extracts had pH of 5 which is suitable for skin care application. HRR possessed the highest percentage yield (12.07%) while HGR and EGA showed the lowest (1.93%). The hexane extracts from both green and roasted coffee beans showed higher percentage yield than ethanolic extracts. This might be due to the non-polar property of hexane that could extract most of the lipid contents from the coffee bean. Additionally, the roasted coffee bean showed higher lipid contents than the green coffee bean in both species corresponding to their percentage yield (Farah, 2012).

Determination of total phenolic content

Total phenolic contents of all the extracts were determined by Folin-Ciocalteu assay. The total phenolic contents of both ethanolic green and roasted coffee extracts were statistically different. From the results, ERR presented the highest phenolic content (287.54 mg gallic acid/g extract) followed by EGA, EGR and ERA, respectively (255.99, 238.94 and 90.95 mg gallic acid/g extract) as shown in Table 2. In contrast, for hexane extracts, their total phenolic contents were not detectable. The results showed that total phenolic content of the green coffee bean extract was significantly higher than roasted coffee beans, except ERR. This might be due to auto-oxidation or degradation during the roasting process, leading to the decreased of polyphenol level in roasted coffee beans (Cheong et al., 2013). Generally, many research papers presented that the phenolic compounds were the good free radical scavenger. In addition, previous studies showed that coffee bean contained many polyphenolic compounds such as chlorogenic acid, mangiferin and hydroxycinnamic acid esters (Vignoli et al., 2011; Campa et al., 2012; Moreira et al., 2013). The major phenolic acid in all coffee samples was chlorogenic acid (Cheong et al., 2013). Therefore, the extracts that revealed a high total phenolic content tends to present a high level of antioxidant activity.

The determination of antioxidant activities

The coffee bean extracts' antioxidant activity was evaluated by DPPH, ABTS and lipid peroxidation inhibition (linoleic acid) assays when compared with

Table 2. Total phenolic contents and antioxidant activities of coffee bean extracts evaluated by DPPH, ABTS and lipid peroxidation inhibition assays.

Samples		**Total phenolic (mg gallic acid/g extract)**	**IC_{50} (mg/ml)**		
			DPPH assay	**ABTS assay**	**Lipid peroxidation inhibition assay**
Hexane extract	Green Arabica (HGA)	ND	6.970 ± 0.16^{a}	0.790 ± 2.29^{a}	8.240 ± 0.01^{a}
	Roasted Arabica (HRA)	ND	4.880 ± 0.12^{b}	1.920 ± 0.80^{b}	10.030 ± 0.01^{b}
	Green Robusta (HGR)	ND	10.340 ± 0.51^{c}	5.010 ± 1.29^{c}	9.786 ± 0.01^{c}
	Roasted Robusta (HRR)	ND	5.750 ± 0.32^{d}	1.940 ± 0.08^{d}	8.230 ± 0.02^{d}
Ethanolic extract	Green Arabica (EGA)	255.99 ± 2.05^{a}	0.050 ± 0.01^{e}	0.016 ± 0.01^{e}	1.246 ± 0.85^{e}
	Roasted Arabica (ERA)	90.95 ± 1.93^{b}	0.180 ± 0.01^{f}	$0.024 \pm 0.01^{e,f}$	0.405 ± 0.02^{f}
	Green Robusta (EGR)	238.94 ± 0.44^{c}	0.070 ± 0.01^{g}	0.014 ± 0.01^{e}	2.632 ± 1.71^{g}
	Roasted Robusta (ERR)	287.54 ± 4.30^{d}	0.090 ± 0.01^{h}	$0.023 \pm 0.01^{e,g}$	5.144 ± 0.01^{h}
Natural extracts	Turmeric extract	-	0.040 ± 0.01^{e}	$0.03 \pm 0.04^{f,g}$	2.414 ± 0.52^{i}
	Mangosteen extract	-	0.36 ± 0.00^{i}	0.06 ± 0.50^{h}	2.018 ± 1.00^{j}
Standard	Trolox (µg/ml)	-	0.005 ± 0.19^{j}	0.864 ± 0.01^{i}	0.047 ± 0.01^{k}
	Gallic acid (µg/ml)	-	0.002 ± 0.31^{j}	0.599 ± 0.01^{i}	0.124 ± 0.01^{k}
	Quercetin (µg/ml)	-	0.006 ± 0.18^{j}	0.538 ± 0.03^{i}	0.083 ± 0.02^{k}

ND = Not detectable, mean values with different letters in the same column are significantly different in Tukey's test ($p \leq 0.05$).

natural extracts (turmeric extract and mangosteen extract) and standards: trolox, gallic acid and quercetin. DPPH assay is widely used for testing the ability of compounds that act as free radical scavengers or hydrogen donors. Turmeric extract and mangosteen extract are widely used as active ingredients in anti-aging products due to their antioxidant activity. Therefore, researchers selected these extracts to compare biological activities with coffee bean extracts. The results are shown in Table 2. A lower IC_{50} value revealed a good antioxidant activity. Ethanolic extracts showed the higher antioxidant activity was significantly different from hexane extracts due to the presence of phenolic compounds that could be extracted by a more polar solvent (Prieto and Vázquez, 2014). Therefore, the research focus on the results of ethanolic extracts. Ethanolic green coffee bean extracts showed higher activity than ethanolic roasted coffee bean extracts in the same species that may be related to the higher polyphenol contents, especially chlorogenic acid (Yashin et al. 2013). Chlorogenic acid is a major component in green coffee beans and is reduced by the roasting process. There are many antioxidant experiments which prove that the phenolic compounds were the good free radical scavenger as mentioned above (Sendra, 2009). These results also strongly indicated that phenolic compounds in coffee bean are major contributors to their antioxidant capacity. The results also showed no significant differences in the antioxidant capacity of EGA and turmeric extract. Additionally, the ethanolic extracts of both species revealed a better antioxidant activity than in the mangosteen extract. The results from ABTS assay exhibited the same trend as DPPH assay. The hexane extracts revealed IC_{50} value much significantly higher than the ethanolic extracts. Additionally, the EGA and EGR presented better activity than turmeric extract while all ethanolic extracts presented a significantly higher level of activity than in the mangosteen extract. The results from lipid peroxidation inhibition assay also showed that ethanolic extracts significantly inhibited lipid peroxidation better than hexane extracts. The ethanolic roasted arabica bean extract showed a better activity than green arabica bean extract. This result might be due to the roasted coffee bean containing higher caffeine (lipophilic

Table 3. Percentage inhibition of coffee bean extracts evaluated by mushroom tyrosinase inhibition activity.

Samples		Inhibition (%) (concentration 2.5 mg/ml)	
		L-tyrosine	L-dopa
Hexane extract	Green Arabica (HGA)	13.50±0.01[a]	2.53±0.02
	Roasted Arabica (HRA)	17.15±0.02[b]	12.07±0.02
	Green Robusta (HGR)	12.12±0.05[c]	2.61±0.05
	Roasted Robusta (HRR)	ND	14.43±0.02
Ethanolic extract	Green Arabica (EGA)	44.27±0.01[d]	ND
	Roasted Arabica (ERA)	20.93±0.01[e]	ND
	Green Robusta (EGR)	23.20±0.05[f]	ND
	Roasted Robusta (ERR)	11.17±0.02[g]	ND
Natural extract	Turmeric extract	3.97±0.02[h]	ND
	Mangosteen extract	ND	ND
Standard	Kojic acid (0.25 mg/ml)	92.79±0.23[i]	86.07±0.58
	α-arbutin (0.25 mg/ml)	58.91±0.11[j]	ND
	β-arbutin (0.25 mg/ml)	49.06±1.16[k]	ND
	Ellagic acid (0.25 mg/ml)	ND	70.61±0.83

ND = not detectable, Mean values with different letters in the same column are significantly different in Tukey's test ($p \leq 0.05$).

agent) than the green coffee bean that could better react with linoleic acid and inhibit lipid peroxidation. On the other hand, the ethanolic green Robusta bean extract exhibited high activity than the roasted Robusta bean extract due to synergism effect of phenolic compounds. Moreover, EGA and ERA showed a good anti-lipid peroxidation activity as compared to turmeric and mangosteen extracts. However, all the extracts showed a lower antioxidant activity than the standards. The ethanolic coffee bean extracts revealed good antioxidant activity with different assays as mentioned earlier. They could also inhibit lipid peroxidation which is a major cause of skin aging. Therefore, the ethanolic extracts were selected for further study.

Determination of mushroom tyrosinase inhibition activity

Tyrosinase enzyme plays an important role in melanin synthesis. It can change tyrosine to L-dopa, then convert to dopaquinone and with several polymerization reactions, eumelanin and pheomelanin are formed (Chang, 2009). Compounds that can inhibit tyrosinase enzyme are used as skin brightening agent. The results are shown in Table 3. When tyrosine was used as substrate, EGA revealed the highest activity (%inhibition = 44.27%). HRR, mangosteen extract and ellagic acid showed no activity. The coffee bean extracts presented a higher activity than turmeric extract. However, all the extracts presented a lower activity than kojic acid and arbutin. These indicated that antioxidant compounds might promote the tyrosinase inhibition activity due to their antioxidative synergistic (Chang, 2009). Therefore, the extracts which consist of high amounts of total phenolic compounds possessed a good inhibition to tyrosinase enzyme. In the part of L-dopa substrate, HRR showed the highest percentage of inhibition, whereas the ethanolic extracts showed no activity. Interestingly, the hexane extracts could inhibit tyrosinase enzyme in the step of converting L-dopa to dopachrome while α-arbutin and β-arbutin could not. It may be due to the components of triglycerides in the hexane extracts that are binding with some sites of the tyrosinase enzyme (Chang, 2009). It could be concluded that the ethanolic coffee bean extracts were the alternative ingredients in whitening products or mixing with other brightening natural ingredients.

The effect of coffee bean extracts on cell viability

The cytotoxicity of coffee bean extracts was measured in RAW 264.7 cells using MTT assay. Percentage of cell viability between samples and the control at the same concentration (100 mg/ml) is shown in Figure 1. Caffeine and chlorogenic acid were used as controls. The results revealed that all extracts showed no toxicity on cells including caffeine, whereas chlorogenic acid presented only 61.37% of cell viability due to its acidity. Additionally, the extracts showed a higher percentage of cell viability than 100 which is in accordance with the effect of caffeine on cell viability. This result improves the assertion that the selected extracts are safe and can be developed as skin care products.

Determination of TLC chromatogram and the identification of chemical components of extracts using HPLC

The ethanolic extracts showed good biological activities,

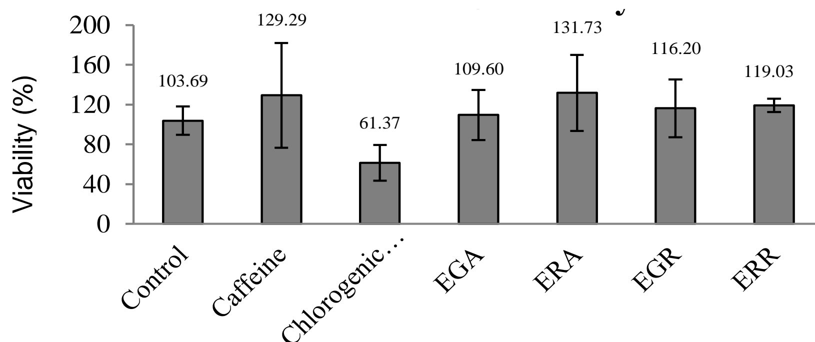

Figure 1. Percentage viability of RAW 264.7 cells treated with extracts and detected by MTT assay.

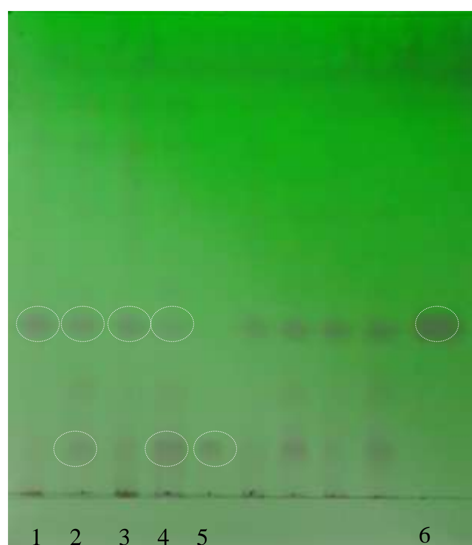

Figure 2. TLC chromatogram detected with UV 254 nm (1 = roasted Robusta (ERR), 2 = green Robusta (EGR), 3 = roasted Arabica (ERA), 4 = green Arabica (EGA), 5 = chlorogenic acid (R_f = 0.1), caffeine (R_f = 0.35).

therefore they were selected to further analyze major constituent by TLC. Coffee bean extracts, caffeine and chlorogenic acid were spotted on Merck Silica gel 60 F254 plate and developed with the mobile system of toluene: ethyl acetate : water : formic acid (15:90:5:5). Then, the chromatograms were detected under a short UV wavelength (246 nm). The TLC plates emitted green light where the compounds absorbed the light, and indicated as the dark areas. All the coffee bean extracts showed a deep dark spot with the same retardation factors with caffeine (R_f = 0.35) as shown in Figure 2 and Table 4. In addition, the chlorogenic acid, EGR and EGA showed the dark spot at the same distance (R_f = 0.1).

According to the results from TLC, caffeine was found in all extracts, whereas chlorogenic acid could be found only in the green coffee bean extracts due to the low amount in roasted coffee bean extracts. These results are related to the previous study which stated that caffeine was found in both green and roasted coffee beans. The previous study also indicated that chlorogenic acid was found in a higher amount in green coffee bean than roasted coffee bean. This may be due to its degradation by heat (Farah, 2012). It could be assumed that caffeine and chlorogenic acid are key compounds in coffee bean that serve as antioxidant and anti-tyrosinase ingredient.

The ethanolic extracts were evaluated by HPLC using caffeine and chlorogenic acid as reference standards. The retention time of caffeine reference was 8.514 min, while retention time of chlorogenic acid was 9.450 min. The HPLC chromatogram of ERR and ERA showed a peak of caffeine, whereas EGR and EGA presented

Table 4. Retardation factors of extracts and standards.

Samples		R_f (cm)
Ethanolic extract	Green Robusta (EGR)	0.1, 0.35
	Green Arabica (EGA)	0.1, 0.35
	Roasted Robusta (ERR)	0.35
	Roasted Arabica (ERA)	0.35
Standard	Chlorogenic acid	0.1
	Caffeine	0.35

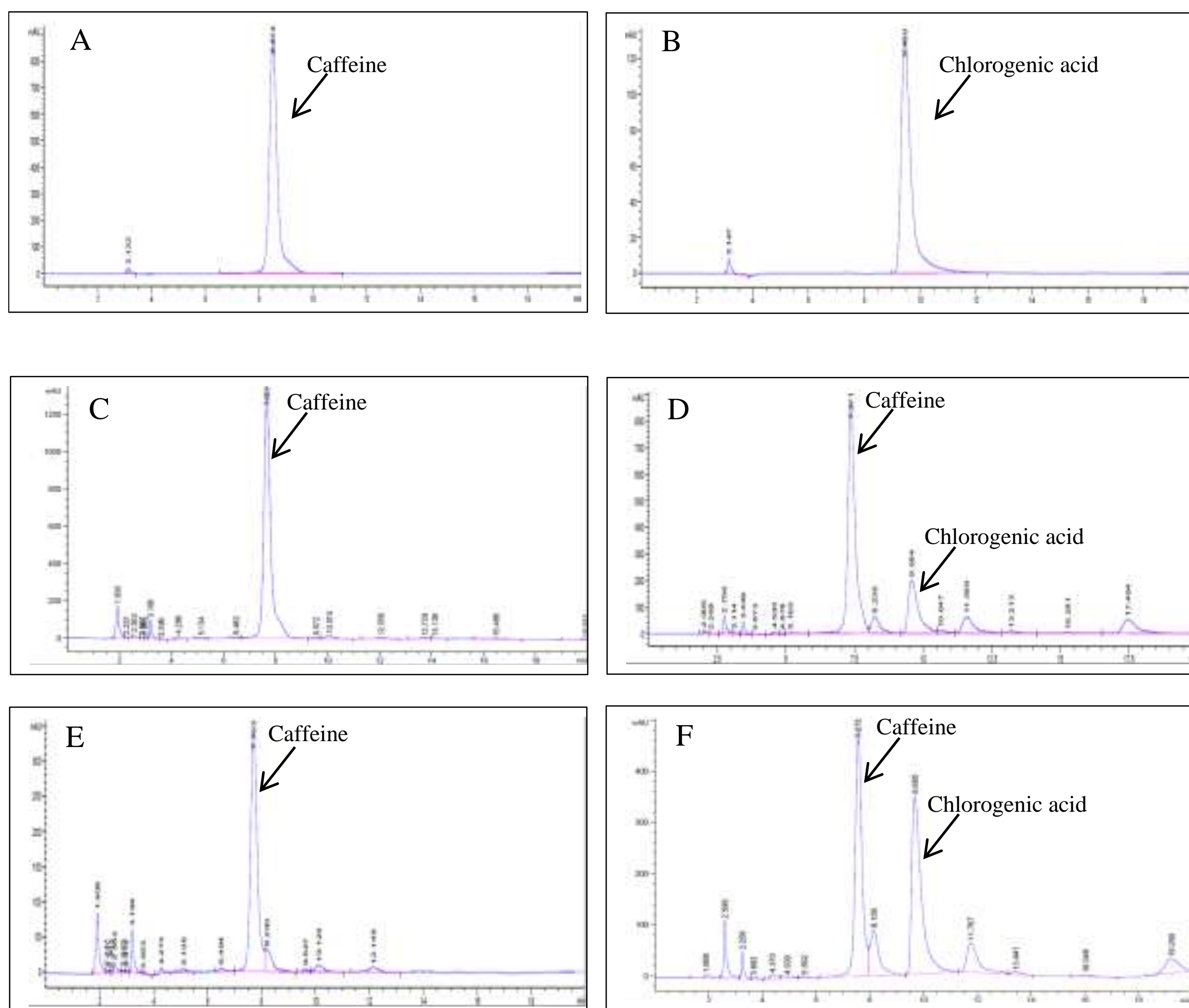

Figure 3. HPLC chromatograms of caffeine (A), chlorogenic acid (B), ERR (C), EGR (D), ERA (E) and EGA (F).

both peaks of caffeine and chlorogenic acid as shown in Figure 3. The HPLC chromatograms are related to the results from TLC chromatogram. The roasted coffee bean extract loss of chlorogenic acid may be due to high temperature during the roasting process.

The stability of coffee bean extracts

The ethanolic extracts were kept in various storage conditions: room temperature (RT), the room temperature in the dark condition (DRT), 4 and 45°C for 3 months

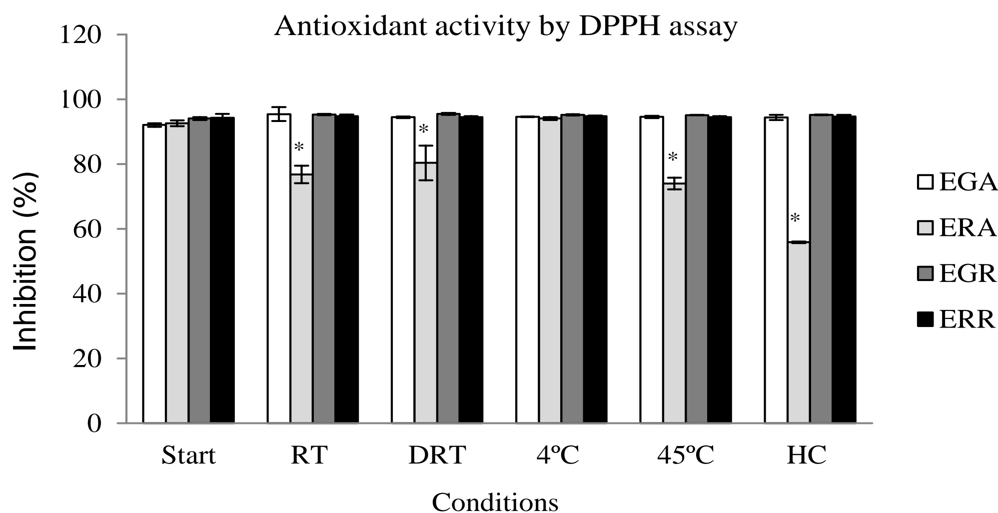

Figure 4. Percentage inhibition of coffee bean extracts by DPPH assay before and after the stability test (*=significant at P<0.05 compared between different conditions of each extract).

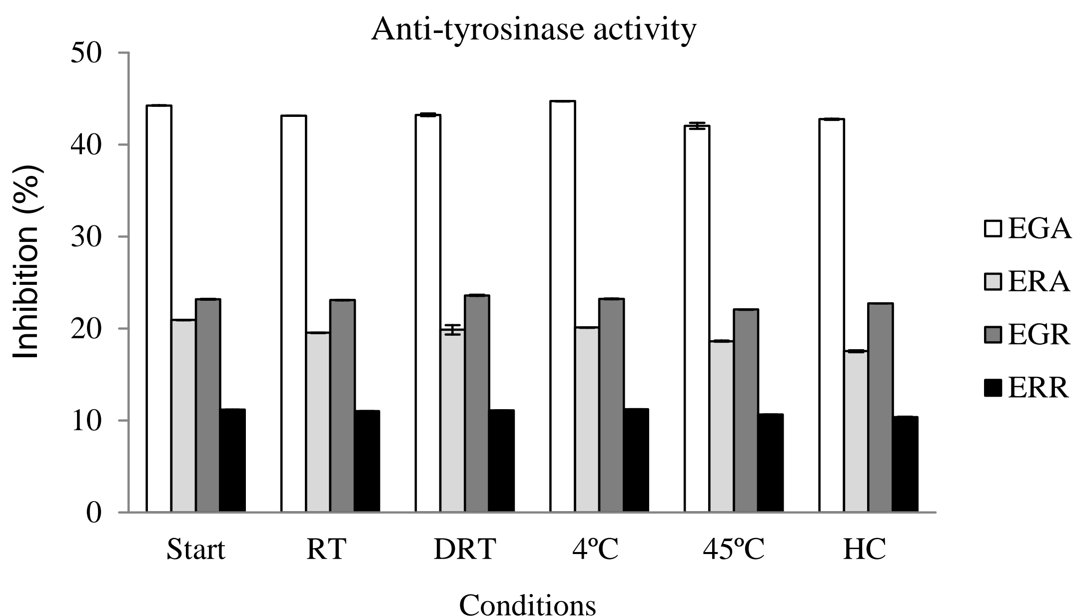

Figure 5. Percentage inhibition of coffee bean extracts by mushroom tyrosinase inhibition activity assay before and after stability test (*=significant at P<0.05 compared between different conditions of each extract).

and heating-cooling (HC) for 6 cycles. After stability test, the extracts were analyzed by DPPH assays and mushroom tyrosinase inhibition activity assay. The results are shown in Figures 4 and 5. The percentage of inhibition of green Arabica (EGA), green Robusta (EGR) and roasted Robusta (ERR) extracts did not change after being stored in all conditions. Whereas, roasted Arabica (ERA) extract showed a significant decrease in the percentage of inhibition ($P<0.05$) after being stored at all conditions except 4°C. In contrast, the results from the mushroom tyrosinase inhibition activity assay showed that the percentage of inhibition did not change after being stored at various conditions. The results are related to their chemical compositions. Previous report indicated that Arabica coffee beans consist of coffee oil (cafestol and kahweol), triglycerides, fatty acids and tocopherol that are sensitive to heat, light and oxygen (Farah, 2012). Therefore, these compounds degrade after a stability test leading to a decrease in the antioxidant activity.

Therefore, the extracts should be kept to avoid light

and heat to protect the degradation of active compounds.

Conclusion

In this study, the green and roasted coffee bean extracts from Arabica and Robusta beans were extracted with hexane and then followed by ethanol with maceration. The hexane extracts showed higher percentage of yields than in the ethanolic extracts; this may be due to high lipid contents. However, the ethanolic extracts possessed higher total phenolic contents and an enhanced level of antioxidant activity than in the hexane extracts. All the extracts except HRR could inhibit tyrosinase activity when using L-tyrosine as a substrate, whereas the hexane extracts showed anti-tyrosinase activity when L-dopa was used as a substrate. Antioxidant and anti-tyrosinase activities of extracts are related to the amount of caffeine and polyphenol contents. The higher caffeine and polyphenol contents generated higher biological activities. The ethanolic extracts that indicated good biological activities and non-toxicity were chosen for a further study. From TLC and HPLC chromatograms, the selected ethanolic extracts consisted of caffeine, while chlorogenic acid was found only in the green coffee bean extracts. The extracts also possessed good activities after being stored at various conditions for 3 months. Therefore, the ethanolic coffee beans are a promising source of natural antioxidant and anti-tyrosinase agent, and should be further developed into cosmeceutical products such as anti-aging or brightening products.

Conflict of interest

The authors have not declared any conflict of interest

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