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Evaluation of extracts of leaf of three *Ficus deltoidea* varieties for antioxidant activities and secondary metabolites

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ABSTRACT

Present study aimed to investigate alcoholic and aqueous extracts of three varieties of *Ficus deltoidea* for antioxidant activity using *in vitro* models such as free radical scavenging activity, reduction power of iron (III), superoxide anion ($O_2^{\cdot-}$) scavenging, xanthine oxidase (XOD), nitric oxide ($NO^{\cdot}$) and lipid peroxidation. The total polyphenols, flavonoids and tannins were estimated using colorimetry in order to investigate their correlation with antioxidant activities. Alcoholic and aqueous extracts of different varieties of the plant exhibited different radical scavenging activities in different models ($P<0.05$). Both the types of extracts displayed high antioxidant activity in DPPH and superoxide anion scavenging models, and the activity was comparable to quercetin, rutin, butylated hydroxyanisole (BHA), ascorbic acid and allopurinol. A correlation was observed between antioxidant activity and content of total polyphenols, flavonoids and tannins. The results of this study indicate that the extracts of *Ficus deltoidea* possess promising antioxidant activity.

Keywords: *Ficus deltoidea; Flavonoids;* Polyphenols; Tannins; Antioxidant

INTRODUCTION

Ficus deltoidea Jack (Moraceae) is found in the South East Asian countries and the plant has several varieties (1, 2). The leaves of the plant are used traditionally for treating diabetes, high blood pressure, heart problems, gout, diarrhea, pneumonia and skin diseases (3). The decoction of leaves is also used by women after giving birth to improve blood circulation and regain body strength. Some ethnic groups in East Malaysia consume the plant as a herbal tea for anti-aging and young appearance.

The leaves of the plant are widely used to prepare herbal medicine but the data on the chemical composition of *Ficus deltoidea* is not available. Similarly, till date only two reports, blood glucose lowering effect and antinociceptive effect, are reported (4, 5). To the best of our information, the plant has not been investigated for antioxidant activity and estimation of total content of polyphenolics, flavonoids and tannins.

Therefore, present study aimed to investigate methanol and aqueous extracts of leaves of three varieties of *Ficus deltoidea* for *in vitro* antioxidant activity using different models and analyze the extracts for the estimation of total polyphenolics, flavonoids and tannins.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteau reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, catechin, gallic acid, butylated hydroxylanisole (BHA), quercetin (QTN), ascorbic acid, xanthine oxidase from buttermilk (5U), xanthine,

nitrobluetetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA), allopurinol, apigenin, sodium nitroprusside, sulphanilic acid and N-napthyl ethylenediamine dihydrochloride were purchased from Sigma-Aldrich GmbH, Steimheim, Germany.

Plant material

Three varieties of *Ficus deltoidea* leaves were selected in this study namely *Ficus deltoidea: F. D. var. terengganuensis* (2 samples labeled as FDT1 and FDT2), *F. D. var. angustifolia* (FDA) and *F. D. var. deltoidea* (FDD). Sample of FDT1 was obtained from Malai Herbal Tea, Malaysia. FDT2 and FDA were collected from Terengganu and Selangor, respectively. Sample of FDD was purchased from Nutreeherbs Sdn. Bhd. All the samples were authenticated by Ms Zainon Abu Samah, a botanist from Medicinal Plants Program, Biotechnology Division, Forest Research Institute Malaysia (FRIM) where voucher specimens having numbers FRI 48988 for FDT2 and FRI 54761 for FDA were deposited.

Preparation of the extracts

Dried and ground leaves were extracted with methanol using Soxhlet extractor for 17 hours to get methanol (M) extracts. The extracts were dried in vacuo at 40°C. To prepare aqueous (W) extracts, dried ground and pulverized leaves were macerated with water at 50°C for 3 h and the procedure was repeated twice. The extracts were filtered and dried freeze dryer.

Determination of total polyphenolics

Total polyphenolic content was determined using Folin-Ciocalteu reagents according to the method of Schalbert *et al.* (6), and Jung *et al.* (7), briefly described as 1.25 ml diluted Folin-Ciocalteu reagent (1ml Folin-Ciocalteu : 9 ml H_2O) was mixed with 0.25 ml extract solution (500 ppm in 80% MeOH). After 3 minutes, 1 ml of 75 g/L sodium carbonate (Na_2CO_3) was added to the reaction mixture and incubated for 1 h at room temperature to measure the absorbance at 760 nm. Gallic acid in different concentrations was used as a standard to construct calibration curve. All the samples and the standards were analyzed in triplicate. Total polyphenolic content was estimated as gallic acid equivalents (GAE) and expressed as mg gallic acid per g extract.

Determination of total flavonoids

The total flavonoid content was determined using the colorimetric method of Woisky and Salatino (8) and Jung *et al.* (7), briefly described as 1.5 ml of 2% aluminium trichloride ($AlCl_3$) was added to 1.5 ml extract solution (500 ppm in 80% MeOH). After incubation of 1 hour at room temperature, the absorbance was measured at 420 nm against a blank. QTN was used as a standard to construct the calibration curve. The amount of total flavonoid was expressed as quercetin equivalents (QE, mg quercetin/g extract). All the samples and the standards were analyzed in triplicate.

Determination of total condensed tannins

The content of condensed tannins was estimated according to the method of Heimler *et al.* (9), briefly described as 2 ml of vanillin solution (1 g vanillin/100 ml concentrated sulphuric acid) was added to 1 ml of extracts solution (500 ppm, 80% MeOH). After incubating for 15 min at room temperature, the absorbance was measured at 500 nm against a blank. The amount of total tannins was expressed as catechin equivalents (CE, mg catechin/g sample). All the samples and the standards were analyzed in triplicate.

Antioxidants evaluation DPPH radical-scavenging activity

The antiradical activity of the extracts was evaluated according to the method of Marwah *et al.* (10), briefly described as 1 ml methanolic solution of 1 mM DPPH was added to 1 ml methanolic solution of the extract (500 ppm) in dark. The reaction mixture was allowed to stand for 15 min at ambient temperature and the absorbance was measured at 520 nm against methanol as a blank. Methanolic solution of DPPH was used as a control. All the samples were analyzed in triplicate. Ascorbic acid, QTN and BHA was used as reference free radical scavengers. The percentage of the DPPH radical scavenging activity was calculated using the following equation.

Percentage inhibition (%IP) $= [1 - (A_s/A_c)] \times 100$

Where A_c is the absorbance of the control and A_s is the absorbance of the tested sample.

Superoxide anion scavenging activity:

Measurement of superoxide anion radical scavenging activity by xanthine/xanthine oxidase model was performed as described by McCord and Fridowich (11) with slight modification, briefly described as the reaction mixture was prepared by 1 ml of 50 mM sodium carbonate (pH 10.2), 0.1 mM EDTA, 50 mM NBT, 2.5 mM xanthine, XOD and test solution (extracts/standards). The reaction was started by adding 1 µl of XOD to the reaction mixture and the tube was incubated at 25 °C. A blank solution was prepared without adding enzyme while a control was prepared without adding the extract or standard solution,

which was replaced by equivalent amount of ethanol. After the incubation of 20 min, the absorbance was measured at 560 nm. Allo and QTN were used as standards. The percentage of superoxide anion scavenging activity of the extracts and standards was calculated using the following equation.

Superoxide anion radical scavenging activity (%) = [(A_c – A_s) / A_s] x 100

Where, A_c is the Absorbance of the control and A_s is the absorbance in the presence of extracts and standards.

Xanthine oxidase activity:

The xanthine oxidase activity using xanthine as a substrate was evaluated by measuring the formation of uric acid at 295 nm as described by Ferraz Filha *et al.* (12) and Sweeney *et al.* (13) with slight modification. The primary function of the xanthine-xanthine oxidase system is to oxidize xanthine or hypoxanthine to uric acid. The assay mixture was prepared by 0.5 ml of sample solution (100 ppm), 1.3 ml of 0.05 M phosphate buffer (pH 7.5) and 0.2 ml of 0.04 U/ml enzyme solutions. After pre-incubation of the mixture at room temperature (25°C) for 10 min, the reaction was initiated by adding 1.5 ml of xanthine solution. After incubation of the assay mixture for 30 min at room temperature, the absorbance was measured at 293 nm against a blank that did not contain the enzyme. A control experiment was performed in the absence of the extracts solution. Allo (100 ppm), a known inhibitor of xanthine oxidase, and QTN (100 ppm) were used as standards. Xanthine oxidase inhibitory activity was calculated using the following equation and the activity was expressed as percentage inhibition of xanthine oxidase.

% inhibition of xanthine oxidase = [(A-B)-(C-D) / (A-B)] x 100

Where A is the activity of XO without test material (total uric acid production), B is the blank of A without enzyme, C is the enzyme activity with the test material (extract/standard) and D is the blank of C without the enzyme.

Reducing power by iron (III) to iron (II):

The reducing power of the extracts was determined according to the method described by Choi *et al.* (14) and Ardestani and Yazdanparasti, (15), briefly, 2.5 ml of the extracts and standard dissolved in the 50% MeOH (1000 ppm) was mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferricyanide, $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. Then 2.5 ml of 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 3000 rpm for 10 min. A 2.5 ml aliquot of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml of 0.1 % ferric chloride, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates high reducing power.

NO assay:

The nitric oxide radical scavenging inhibition assay was performed according to a method described by Rai *et al.* (16). In this experiment, the reaction mixture containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml phosphate buffer saline and 1 ml of the test solution (1000 ppm) was incubated at 25 °C for 150 min. For control, an equivalent amount of solvent used to dissolve the extract/standards was added. After the incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulphanilic acid reagent (0.33 % in 20% glacial acetic acid). The mixture was allowed to stand for 5 min for completing diazotization. Then 1 ml of 0.1 % N-napthyl ethylenediamine dihydrochloride was added and the reaction mixture was allowed to stand for 30 min at 25 °C to measure the absorbance at 540 nm against the corresponding blank solution. Gallic acid solution was used as a positive control.

Lipid peroxidation inhibition assay:

The total antioxidant activity of the methanol and water extracts was measured by linoleic acid method as described by Mitsuda *et al.* (17), briefly, 4 ml of extracts and standards solution (1000 and 200 ppm, respectively dissolved in DMSO and buffer solution) was mixed with 4 ml of 0.5 % linoleic acid emulsion, 8 ml phosphate buffer (0.05, pH 7) and 4 ml H_2O. The reaction mixture was incubated at 40 ±1 °C in the dark up to 13 days to accelerate the peroxidation process. The degree of oxidation was measured at day 7 and 13 according to the thiocyanate method by sequentially adding 4.7 ml of 75% ethanol, 0.1 ml 30% ammonium thiocyanate and 0.1 ml ferrous chloride (0.02 M in 3.5% HCl) to 0.1 ml of the reaction mixture. After 3 min, following the color development with $FeCl_2$ and thiocyanate, the thiocyanate value was measured at 500 nm. The percentage inhibition of lipid peroxidation in linoleic emulsion was calculated using the following equation.

Inhibition of lipid peroxidation (%) = (1 – As/Ao) x 100

Where A_s is the absorbance at 500 nm in the presence of sample and A_o is the absorbance of the control.

Statistical analysis

All the samples and the standards were analyzed in triplicate and the results were averaged. The data was analyzed by analysis of variance, one way ANOVA, with multiple comparisons by LSD and $P< 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Formation of radicals and reactive oxygen species (ROS) is a normal physiological process and body has antioxidant defense system against this oxidative stress. Imbalance between oxidative stress and antioxidative defense system leads the formation of ROS that may damage lipids, proteins, DNA and carbohydrates leading to various diseases (18, 19, 20). Antioxidants can protect the body from free radicals and ROS, hence prevent the progression of many chronic diseases (21, 22, 23). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyl anisole (BHA), propyl gallate and tert-butylhydroquinone (TBHQ) are widely used. BHA and BHT are reported to have certain safety concerns (24) due which, the demand of naturally occurring antioxidants is increasing Different antioxidant compounds have different radical scavenging activities against various reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O•) or hydroxyl radical (•OH). In present study, the antioxidant activities of all extracts were evaluated using six *in vitro* models such as 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical scavenging, superoxide anion scavenging, xanthine oxidase inhibition, reduction power capability by iron (III), NO inhibition and lipid peroxidation.

The results of antioxidant activity of the extracts and the standards by DPPH assay are presented in (Figure 1). The activity of the extracts such as FDT1W, FDAM, FDAW and FDDW and the standards was significantly different (*P<0.05*). These results indicated that all the extracts except FDT1W, FDAW and FDDW showed antioxidant activity comparable to that of QTN, rutin, BHA and ascorbic acid.

DPH is a useful reagent for investigating the free radical activities of phenolic compounds. It is used for the evaluation of antioxidant capacity in a short time and frequently applied for testing food products (25, 26). The method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen-donating antioxidants, which results in the formation of non-radical form, DPPH-H. BHA, QTN and ascorbic acid were used as reference radical scavengers.

The results of antioxidant activity by SOD assay shown in (Figure 2) indicated that the activity of all the extracts and the standard was different (*P<0.05*). The results of superoxide anion scavenging activity of the extracts using xanthine/xanthine oxidase model shown in (Figure 3) indicated that the activity of all methanol extracts was different from aqueous extracts. The activity of all the extracts was significantly lower than the activity of the standards, allopurinol and QTN (*P<0.05*).

The extracts were also evaluated for their ability to scavenge superoxide anion radicals ($O_2^{\bullet}$), generated by the xanthine/xanthine oxidase system during the formation of uric acid from the oxidation of xanthine by xanthine oxidase. Superoxide anion radicals are oxygen-centered radicals, which play an important role in the formation of ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen to induce the oxidative damage (27, 28). Therefore, the antioxidative activities of the extracts were also evaluated for their ability to inhibit the formation of uric acid. QTN exhibited the inhibition of uric acid production by 86.17%, which was almost equal to the inhibitory effect of allopurinol. However, all the extracts were ineffective to inhibit the production of uric acid.

In the reducing assay, the yellow color of the test solution changes to various shades of green to blue

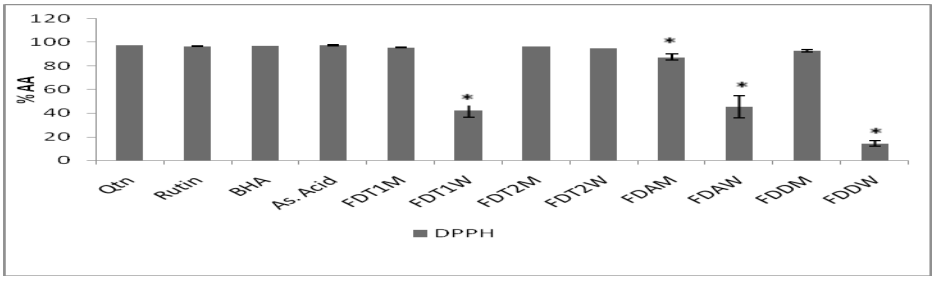

Figure 1: % antioxidant activity of standards, different extracts of leaves of three varieties *Ficus deltoidea* by DPPH model, FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; QTN (quercetin); BHA (butylated hydroxylanisole); Ac. acid (ascorbic acid); * significant difference of samples as compared to all the standards (P<0.05).

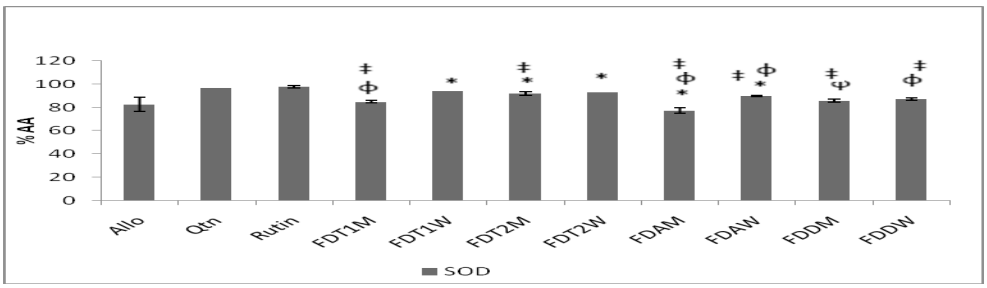

Figure 2: % antioxidant activity of standards, different extracts of leaves of three vareities *Ficus deltoidea* by super oxide dismutase (SOD) assay, FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; Allo (allopurinol); QTN (quercetin); *, ‡ and ф (significant difference of samples as compared to Allo, QTN and Rutin, respectively (P<0.05).

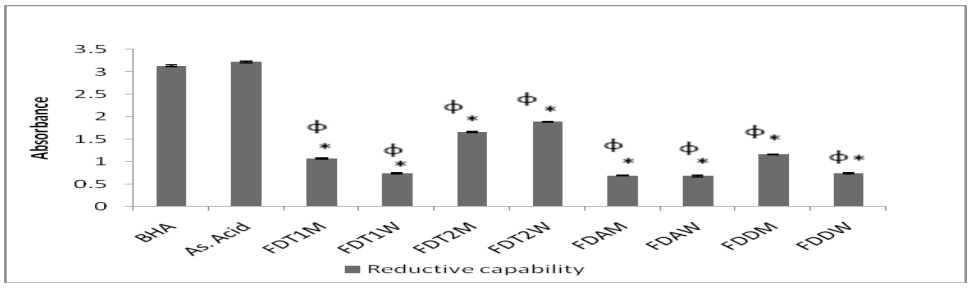

Figure 3:% antioxidant activity of standards, different extracts of leaves of three vareities *Ficus deltoidea* by xanthine oxidase (XOD) assay, FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; Allo (allopurinol); QTN (quercetin); * and ф (significant difference of samples as compared to Allo and QTN, respectively (P<0.05).

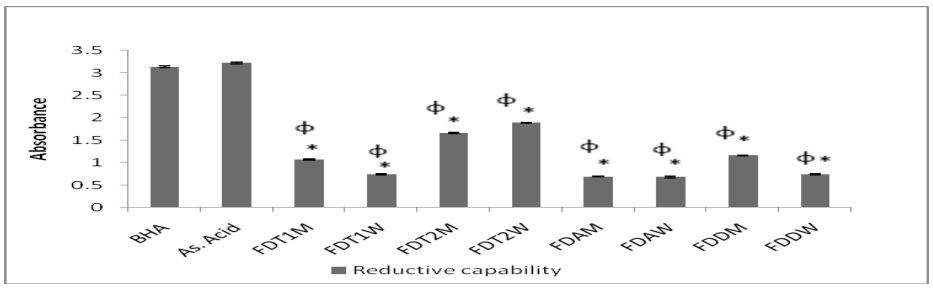

Figure 4: Reduction capbility of standards and different extracts of leaves of three vareities *Ficus deltoidea* from ferric(III) to ferric (II), FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; Allo (allopurinol); BHA (butylated hydroxylanisole); Ac. acid (ascorbic acid); * and ф (significant difference of samples as compared to GA (P<0.05).

depending on the reducing power of the extracts and the standards. The presences of antioxidant substances cause the reduction of Fe^{3+}/ferricyanide complex to ferrous form. The formation of ferrous can be measured by monitoring the Perl's Prussian blue color at 700 nm. The results of the reducing power of the extracts and the standards are shown in (Figure 4). These results indicated that the absorbance of the extracts was slightly lower than that of the reference compounds (*P<0.05*). All the extracts showed the capability of acting as electron donors that can react with free radicals to terminate radical chain reaction.

Nitric oxide radical (NO•) is a very labile molecule generated in tissues by specific nitric oxide synthase, which metabolizes carginine to citrulline with the formation of NO• via a five electron oxidative reaction (28). In addition, under oxidative stress, NO• may reacts with other reactive species to produce much more toxic reactive nitrogen species (RNS) and ROS (28). The scavenging effect of the extracts of *Ficus deltoidea* leaves was evaluated against this radical. In this experiment, diazonium ions were produced when acidified nitrite reacts with suphanilic acid and these diazonium ions form a pink chromophore when reacts with N-naphthyl ethylenediamine dihydrochloride. The percentage inhibition of scavenging of NO radical of the extracts and gallic acid is presented in (Figure 5). All the extracts showed the lower NO radical scavenging activity but activity was comparable to that of gallic acid solution (*P<0.05*). The potential of the extracts in scavenging the nitric oxide was found to be in the order FDT2W > FDAW >FDDW > FDT1W >FDT2M >FDAM >FDT1M >FDAM.

Lipid peroxidation affects the nutritive values of the food and also may cause diseases. The lipid peroxidation inhibition activities of the extracts were measured using ferric thiocyanate (FTC) test which determine the amounts of peroxide produced from the peroxidation of linoleic acid. The results of the peroxidation inhibition capacity of the extracts and the reference compounds are presented in (Figure 6). The difference of activity between different extracts and the standards was significant (*P<0.05*). These results indicated that the percentage inhibition of all the extracts and the reference compounds decreased during incubation. FDT1M, FDT2M, FDT2W, FDAM, FDDM and FDDW demonstrated inhibition of peroxidation on day 7th of incubation as 76.64, 77.29, 76.65, 76.76 and 76.29, respectively, while the activity of BHT, QTN and rutin was 88.48, 83.62 and 67.12, respectively. Furthermore, the extracts and the standards demonstrated decreased percentage inhibition on the 13th day.

The content of total polyphenolics, flavonoids and tannins in various extracts is shown in (Table 1). The results showed that methanol extracts contain higher polyphenolics than the water extracts. FDT2M showed the higher content of polyphenolics and tannins (172 and 942.15 mg/g, respectively), while FDAM showed the high content of flavonoids 86.85 mg/g. The results indicated that *Ficus deltoidea* var. *terengganuensis* was rich in polyphenolics, flavonoids and tannins as compared to other varieties. The correlation of total polyphenols, flavonoids and tannins, and antioxidant activity was observed. Plant polyphenols are known to have antioxidants properties (29) and their use may prevent oxidative stress, associated with diseases such as cancer, neurodegenerative and cardiovascular diseases (30, 31). Hence, the total polyphenolic content should be given consideration while preparing antioxidant formulation from the leaves of the plant.

CONCLUSION

In this study, the extracts showed the potential of scavenging the free radicals under *in vitro* conditions by different mechanisms. In conclusion, the study suggested that the high total amount of polyphenols, flavonoids

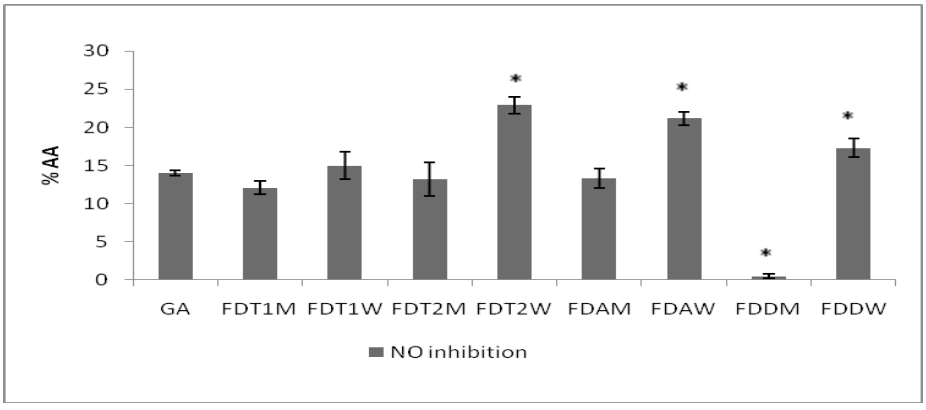

Figure 5: % antioxidant activity of standards and different extrats of leaves of three varities *Ficus deltoidea* by nitric oxide (NO) assay, FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; Allo (allopurinol); GA (gallic acid); * (significant difference of samples as compared to GA (P<0.05).

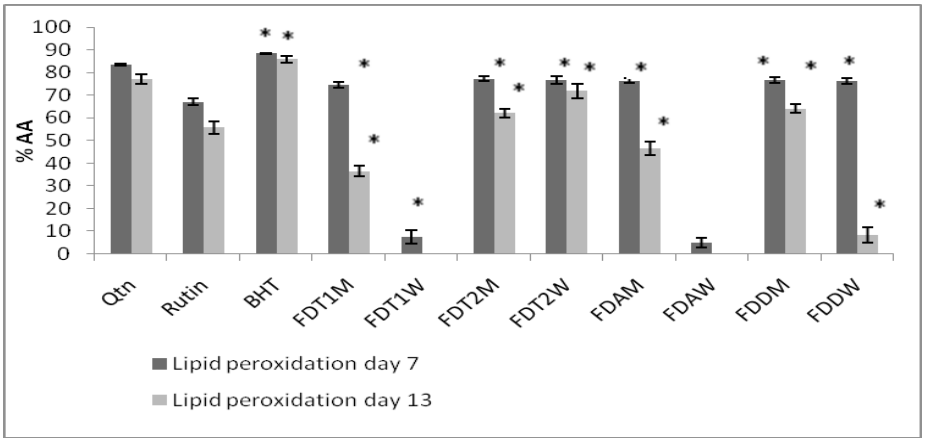

Figure 6: % antioxidant activity of standards and different extrats of leaves of three varities Ficus deltoidea by lipid peroxidation assay at day 7 and 13, FDT1M and FDT2M (methanol extracts of F. D. var. terengganuensis sample 1 and 2); FDT1W and FDT2W (aqueous extracts of F. D. var. terengganuensis sample 1 and 2); FDAM (F. D. var. Angustifolia methanol extract); FDAW (F. D. var. Angustifolia aqueous extract); FDDM (F. D. var. Deltoidea methanol extract). FDDW (F. D. var. Deltoidea aqueous extract; Allo (allopurinol); QTN (quercetin); * (significant difference of samples as compared to QTN, BHT and Rutin (P<0.05).

Table 1. Total amount of polyphenolics, flavonoids and tannins in methanol and water extracts of *Ficus deltoidea* species.

Sample code	Total polyphenolics mg/g	Total flavonoids mg/g	Total tannins mg/g
FDT1M	111.31 ± 0.35	73.49 ± 1.07	497.68 ± 5.97
FDT1W	73.49 ± 0.54	69.58 ± 1.90	218.37 ± 4.22
FDT2M	172.78 ± 0.47	51.49 ± 0.56	942.15 ± 4.08
FDT2W	147.37 ± 2.14	52.54 ± 1.34	732.69 ± 5.83
FDAM	60.59 ± 0.96	86.85 ± 0.58	178.63 ± 2.83
FDAW	36.37 ± 0.27	27.35 ± 1.082	96.32 ± 0.75
FDDM	73.42 ± 1.72	42.83 ± 0.61	323.40 ± 6.42
FDDW	43.52 ± 1.10	42.63 ± 0.48	130.30 ± 3.37

and tannins in the extracts is associated with antioxidant activity of the extracts.

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