Interdiscip Toxicol. 2020; **Vol. 13**(3): 112–116.
doi: 10.2478/intox-2020-0015

sciendo

interdisciplinary toxicology

RAPID COMMUNICATION

Cytotoxic profile of different extracts of *M. calabura* fruits and antiadipogenic activity of ethanol extract in 3T3-L1 cells

Mahasenan RENJITH [1,2], Kottayath Govindan NEVIN [3,4]

[1] *School of Biosciences, Mahatma Gandhi University, PD hills PO, Kottayam, Kerala, India*
[2] *Calabura labs, K. S. Puram. Karunagapally PO, Kollam, Kerala, India*
[3] *School of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panagad PO, Kochi, India*
[4] *Centre for Bioactive Substances from Marine Organisms, Kerala University of Fisheries and Ocean Studies, Panagad PO, Kochi, India*

ITX130320A05 • Received: 03 September 2018 • Accepted: 27 November 2020

ABSTRACT

Lipid accumulation is the root cause of obesity, which is a global epidemic metabolic syndrome affecting all populations worldwide. Due to the deleterious hepatotoxic and cardiotoxic effects of various antiobesity drugs, the attention now turns on to various natural products for low toxic therapeutic molecules against lipid accumulation. *Muntingia calabura* is a shrub or tree, whose fruits are edible. This study evaluated the possibility of the ethanol extract from fruits of *M. calabura* as functional food towards reduction of obesity. Anti obesity and cytotoxic activity of this extract was tested in 3T3-L1 preadipocytes. The *in vitro* antioxidant activity was also tested colorimetrically. Results showed that the ethanol extract of *M. calabura* fruits has significant oxygen, hydrogen and DPPH radical scavenging activity *in vitro*. The extract dose-dependently suppressed adipocyte differentiation and lipid accumulation in 3T3-L1 cell line as indicated by changes in cellular lipid levels evaluated by Oil red O staining. Taken together, these results suggest that the ethanol extract of *M. calabura* fruits may have antiobesity and antioxidant effects with the potential for use in obesity management in humans.

KEY WORDS: *Mutingia calabura*; antiobesity; 3T3-L1 cells; cytotoxicity

Introduction

Obesity is a global epidemic metabolic syndrome affecting all populations worldwide by increase in body weight caused by the excessive accumulation of lipids resulting in other serious life-threatening conditions *viz* insulin resistance-related diabetes, hypertension, and dyslipoproteinemia (Kim *et al.,* 2017; Arita *et al.,* 1999; Lumeng *et al.,* 2007). Lipid accumulation, the root cause of obesity, which results in adipogenesis, is characterized by increased triglyceride production and adipocyte differentiation (Soukas *et al.,* 2001). There are various strategies from pharmacological to complementary therapies for management of obesity. The presently available antiobesity drugs act by reducing appetite or by inhibiting fat absorption. These drugs that reduce obesity can also have serious deleterious hepatotoxic and cardiotoxic effects (Lemhadri *et al.,* 2007). Physical activity, lifestyle modifications and the use of medicinal plants can be an alternative practice to reduce obesity as it poses no toxicological treatment and side effects. Natural products isolated from traditional medicinal or non-medicinal plants have always given an exciting opportunity for the development of newer effective therapeutic agents (D'Mello *et al.,* 2011).

Muntingia calabura, belonging to the family *Muntingiaceae,* is a shrub originating from Mexico. The fruits of *M. calabura* are edible and can be eaten raw or processed as jam. Different parts of *M. calabura* are used in traditional medicine for treating headaches, prostate problems, gastric ulcers and respiratory ailments (Mahmood *et al.,* 2014). The present study evaluates the antioxidant activity of different extracts of *M. calabura in vitro* and antiadipogenic activity of its ethanolic extract in 3T3-L1 cells.

Correspondence address:
Dr. Kottayath Govindan Nevin
School of Ocean Science and Technology,
Kerala University of Fisheries and Ocean Studies,
Panangad PO, Kochi, India-682506
E-MAIL: nevinkg@kufos.ac.in

Materials and methods

Chemicals

Fluorescein disodium salt, 2,2-azobis-(2amidinopropane)dihydrochloride (AAPH), 6- hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, Dulbecco's Modified Eagle Medium (DMEM), dexamethasone (DEX), 1-methyl-3-isobutyl xanthine (IBMX), insulin from bovine pancreas (INS), formaldehyde, and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA) were obtained from Sigma-Aldrich (USA). All other solvents used were of highest analytical grade and purchased from local distributors.

Preparation of the extract

Fruits of *M. calabura* (Figure 1) was collected from the outskirts of Bangaluru, India and dried for one week. 1.67 kg of dried fruit was extracted with *n*-hexane to remove the fatty components. The residue obtained was subsequently extracted sequentially with ethyl acetate ethanol and water. The ethyl acetate extract (EAMC), ethanol extract (EMC) and water extract (WMC) were dried in rotary evaporator, weighed and yield was calculated.

Figure 1. Picture of raw and ripe *M. calabura* fruits.

Antioxidant assays

DPPH radical Scavenging activity

The free radical scavenging activity of the extract from *M. calabura* fruit was evaluated *in vitro* using DPPH radical. Various concentrations of extracts (0.91–300 µg/ml) were mixed with 1.0 ml DPPH solution in ethanol (0.8 mM). The mixture was shaken vigorously and left to stand for 30 min in room temperature. After the incubation, absorbance was measured at 517 nm against a reagent blank. Gallic acid was used as standard. The percentage scavenging DPPH radical was calculated according to the equation; % Scavenging = [1–(Ab *sample* / Ab *control*] × 100 (Koleva *et al.,* 2002).

Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity (ORAC) of the fruit extracts from *M. calabura* was evaluated by the method using fluorescein. Briefly, different concentrations of the extract were mixed with 50 µl of fluorescein (0.1 µM) and was incubated for 15 min at 37 °C. After incubation, 50 µl of 2,2'-azobis(2-methylpropionamide)-dihydrochloride (AAPH) solution (60 mM) was added rapidly. The mixture was shaken and the fluorescence was recorded every minute for 100 minutes using a Microplate Reader with 485-P excitation and 535-P emission filters. A blank using phosphate buffer instead of the antioxidant solution and six calibration solution using trolox standard (0, 2, 4, 8, 12, 16 µM) were carried out in each assay. Final ORAC values were expressed as µM trolox equivalent (TE) (Prior *et al.,* 2005).

Hydroxyl Radical Antioxidant Capacity Assay (HORAC)

The protecting ability of the fruit extract from *M. calabura* against formation of hydroxyl radical was measured according to the protocol described earlier. Briefly, different concentration of the extract was incubated with the mixture containing CoF2.4$H_2$0 (4.6 mM), 20 mg picolinic acid (v/v in distilled water) and 170 µl of fluorescein (60 nM, final concentration) in ELISA plate and incubated at 37 °C for 10 min. After incubation, 10 µl H_2O_2 (0.55 M) and 10 µl of Co (II) (230 µM) were added subsequently. The initial fluorescence was measured every minute after shaking. Phosphate buffer solution was used as blank. Gallic acid was used for plotting the standard curve. Final HORAC values for the sample was expressed a µM gallic acid equivalence (GE)/mg (Ou *et al.,* 2002).

Cell culture

3T3-L1 preadipocytes were purchased from National Centre for Cell Sciences (NCCS), Pune, India. 3T3-L1 cells (10×10^4) were seeded in a T-75 cm^2 flask containing DMEM medium in a humidified atmosphere (37 °C) of 5% CO_2 and 95% air. Cells were allowed to grow for 48–72 hrs until 70–80% confluence is reached. Confluent cells were used for further studies.

Cytotoxicity analysis using Sulforhodamine B (SRB) assay

To determine the cytotoxicity activity of different extract of *M. calabura* fruit, 3T3-L1 cells (2×10^4cells/well in 6-well plates) were seeded with DMEM medium and incubated for 24 h at 37°C with 95% air and 5% CO_2 to allow the cells to become semi-confluent. After this period the cells were treated with different concentrations of the extract (0.01–2 mg/ml) and incubated further for 24 h. After the incubation period, the cell monolayers were fixed with 10% (w/v) trichloroacetic acid (TCA) and stained for 30 min with SRB solution (0.04% wt/vol in acetic acid), after which the excess dye is removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in Tris base (10 mM) and the optical density was measured at 510 nm using a microplate reader (Fricker, 1994).

Differentiation of preadipocytes

The confluent adherent 3T3-L1 preadipocytes cells were removed by treating with 0.25% trypsin and 0.03% EDTA. The cells were counted and checked for viability. To induce differentiation, 5000 cells/200 µl of subculturing media were seeded in a 96 well plate and kept in a humidified atmosphere (37 °C) of 5% CO_2 and 95% air. After 48 hrs, the media were removed and 200 µl of fresh media was added to each well. After reaching full confluency cells were treated with 200 µl of adipogenesis-inducing medium (3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (10 µM) and insulin (0.05 mg/ml) in high glucose DMEM media with 10% FBS) including different concentrations of the ethanol extract of *M. calabura* fruits and incubated for 72 hrs. To examine the effect of *M. calabura* extract on adipocyte differentiation in 3T3-L1 cells, the media and the extracts were frequently changed everyday until the end of the experiment (Lee *et al.*, 2017).

Oil red staining

3T3-L1 cells (1×10^4) were cultured in DMEM medium and exposed to different concentrations of *M. calabura* extract (15 µg/ml to 2 mg/ml) for 2 days. After the experimental period, media were removed and the cells washed with PBS and fixed with 10% formalin for 30 min. After two washes with distilled water, the cells were stained with freshly diluted Oil Red O solution (stock: 0.5% in isopropanol; working solution 6 parts of oil red O stock mixed with 4 parts milli Q water). The dye retained in the 3T3-L1 cells was eluted with isopropanol and quantified by measuring the absorbance at 492 nm in a microplate reader. The obesity inhibitory activity of the extract was calculated as % inhibition activity ((B–C) – (S–C) / (BC) ×100. Where, B = Absorbance of differentiated cells. C = Absorbance of blank plate, S = Absorbance of test solution. The experiment was repeated and the oil red staining of the 3T3-L1 cells was visualized under a microscope (Sowndhararajan & Kang, 2013).

Statistics

Statistical analysis was done by using one way analysis of variance using the SPSS program. Values of $p<0.05$ were considered to be statistically significant.

Results and discussion

DPPH free radical scavenging activity of different extracts of *M. calabura* fruits was evaluated. The results showed that ethanol (EMC) and ethyl acetate fraction (EAMC) had higher activity at very low concentration compared to water extract (WMC) (Figure 2). ORAC activity of the all three extracts was found to be similar while HORAC values for EAMC was lower compared to WMC and EMC (Figure 2). Previous reports suggests that plant components like polyphenols, flavonoids and alkaloids have potential ability to quench free radicals and thereby prevent lipid oxidation via a chain breaking reaction. Due to this property, these purified compounds or extract containing these compounds could serve as potential nutraceuticals when ingested along with nutrients (Sowndhararajan & Kang, 2013).

Reports showed that *M. calabura* fruit has low energy value, attractive colour and flavour with several potent bioactive compounds with antioxidant activity,

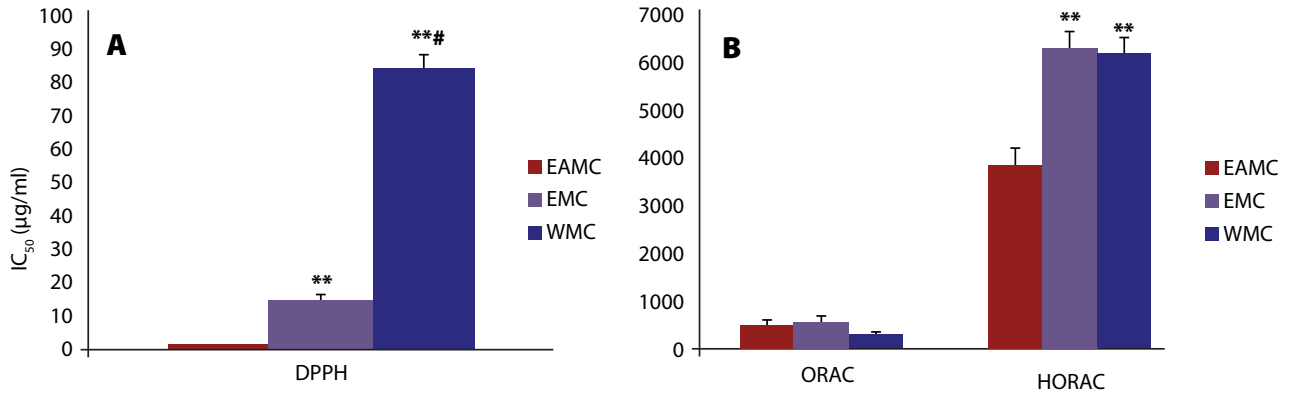

Figure 2. DPPH radical scavenging activity, ORAC and HORAC values of different solvent extracts of *M. calabura* fruit. Values are expressed as ± SEM of three separate experiments. Ethyl acetate extract (EAMC), Ethanol extract (EMC) and Water extract (WMC).**statistically significant compared to EAMC ($p<0.05$), # statistically significant compared to EMC ($p<0.05$). HORAC is expressed as µM gallic acid equivalent/mg extract. ORAC is expressed as µM trolox equivalent/mg extract. A: DPPH radical scavenging activity; B: ORAC and HORAC of different extracts.

viz. terpenes, b-farnesene and dendrolasin, gallic acid, cyanidin-3-O-glucoside as the main phenolic compounds, as well as gentisic acid, gallocatechin, caffeic acid and protocatechuic acid (Pereira *et al.*, 2018).

Cytotoxic activity of different concentrations of the all three extracts was evaluated against 3T3-L1 preadipocytes cells. The results showed that EAMC was cytotoxic to the cells at a very lower concentration, while WMC showed lower but significant cytotoxic activity compared to EAMC. Meanwhile, EMC was found be less cytotoxic to 3T3-L1 cells even at higher concentration tested, compared to other two extracts (Figure 3). Since the cytotoxic activity of EMC was lower, it was used for evaluating the antiobesity activity. To determine the antiobesity activity of the EMC, we tested whether it could prevent the differentiation of preadipocytes 3T3-L1 cells. The preadipocyte cell lines, 3T3-L1 is optimal experimental model to study the differentiation between an immature and mature state, since the physiological and biochemical properties of this cell line are similar to the development of obesity in humans. The results of the present study showed that the different concentrations of ethanol extract of *M. calabura* fruits (EMC) tested had no effect on the viability of preadipocyte 3T3-L1 cells. Treatment of 3T3-L1 cells with EMC affected the morphology, which was evident from the changes occurred from pre-differentiation (spindle-like shape) to fully differentiated cells (round shape) at lower concentrations, while at higher concentration (50 µg/ml) it was found that the 3T3-L1 cells remained as preadipocytes. Cells treated with EMC showed significant concentration-dependent reduction in lipid droplets within the cells, compared to control, as documented by staining with Oil red O. Lower concentration (1.56 µg/ml) showed 15% reduction and higher concentration (50 µg/ml) showed 90% reduction in adipogenesis (Figure 3). Greater increases in adipocyte levels are known to be associated with higher accumulated lipid contents of adipocytes (Poudel *et al.*, 2015). There are several significant reports suggesting the same phenomenon mediated by flavonoids extracted from different fruit extracts (Kim *et al.*, 2012;

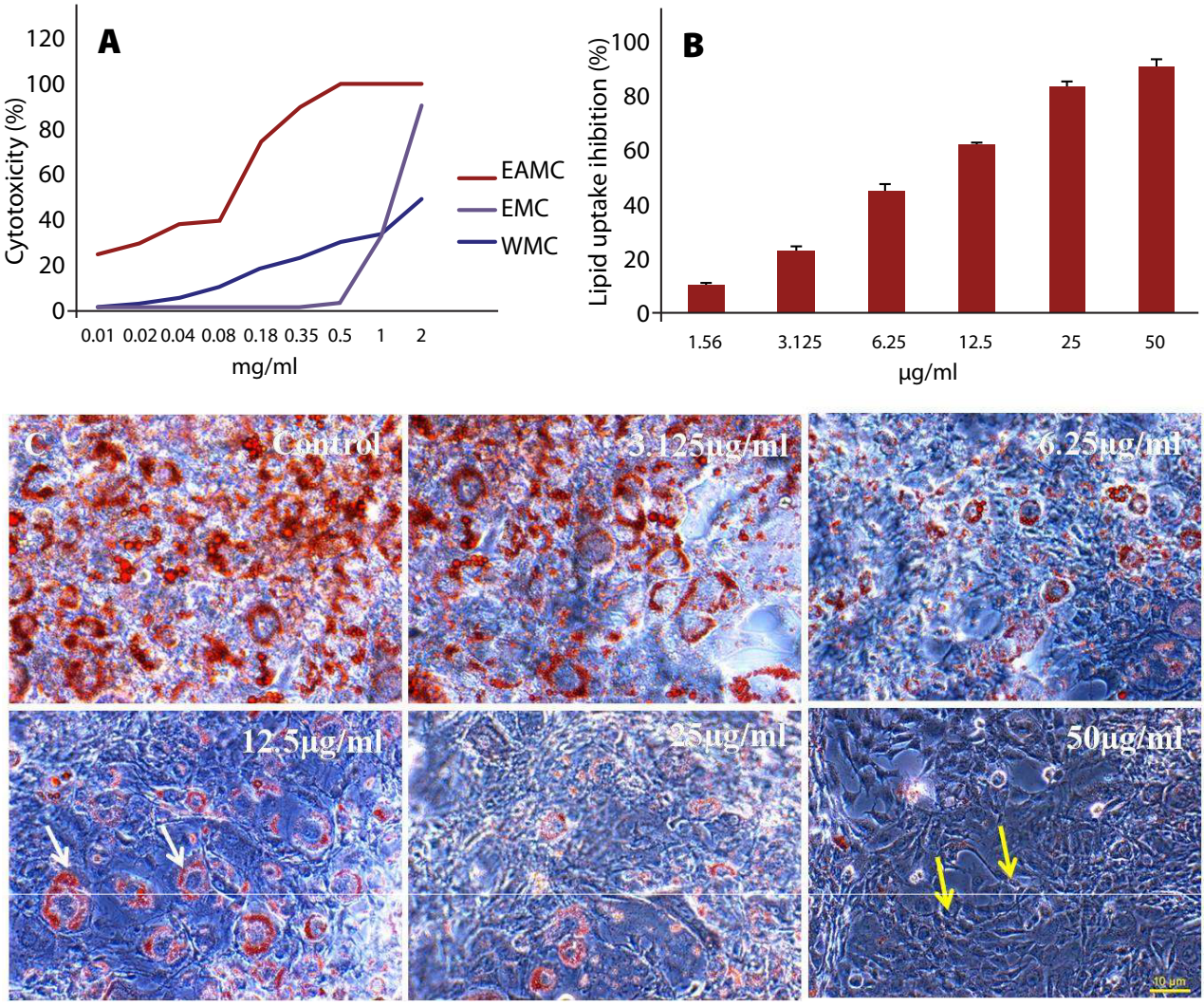

Figure 3. Cytotoxic profile of different extracts of *M. calabura* fruits and anti-adipogenic activity of ethanol extract in 3T3-L1 cells. Values are expressed as ± SEM of three separate experiments. ethyl acetate extract (EAMC), ethanol extract (EMC) and water extract (WMC). A: Cytotoxic activity of different solvent extracts B: percentage inhibition of lipid uptake by 3T3-L1 cells in the presence of *M. Calabura* fruit extract. C: Oil red staining of 3T3-L1 cells treated with *M. calabura* fruit extract. Yellow arrows indicates spindle shaped cells, white arrows indicates rounded cells.

Borah *et al.,* 2019; Duangjai *et al.,* 2018), which antiobesity properties are comparable with EMC.

Conclusion

In the present study, a preliminary evaluation on the antioxidant and the antiobesity inhibitory activity of the ethanol extract of *M. calabura* fruits (EMC) was done. The results showed to be promising since the extract had profound antiadipogenic and antioxidant property *in vitro*. This prompts us to conduct further studies on the molecular aspects of antiobesity property and identification of the active component/s of the extract as well as the possibility of using this extract in an Ayurvedic formulation for antiobesity treatment in humans.

Acknowledgement

This work was done without any external funding

REFERENCES

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa JI, Hotta K, Shimomura I,Nakamura T, Miyaoka K, Kuriyama H. (1999). Paradoxical decrease of an adiposespecific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* **257**: 79–83.

Choi R, Ham JR, Yeo J, Hur J, Park S, Kim M, Lee M. (2017). Anti-obesity property of Lichen *Thamnolia vermicularis* extract in 3T3-L1 cells and diet-induced obese Mice. *Prev Nutr Food Sci* **22**: 285–292.

D'Mello PM, Darji KK, Shetgiri PP.(2011). Evaluation of antiobesity activity of various plant extracts. *Pharmacognosy J* **21**: 56–59.

Fricker SP. (1994). The application of sulforhodamine B as a colorimetric endpoint in a cytotoxicity assay. *Toxicol In Vitro* **8**: 821–822.

Kim G, Kim J, Kim G, Choi S. (2017). Anti-adipogenic effects of Tropaeolum majus (nasturtium) ethanol extract on 3T3-L1 cells. *Food Nutr Res* **61**: 1339555.

Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN. (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem Anal* **13**: 8–17.

Lee JA, Cho Y R, Hong SS, Ahn E K. (2017). Anti-Obesity Activity of Saringosterol Isolated from *Sargassum muticum* (Yendo) Fensholt Extract in 3T3-L1 Cells. *Phytotherapy Res* **31**: 1694–1701.

Lemhadri A, Eddouks M, Sulpice T, Burcelin R. (2007). Anti-hyperglycaemic and Antiobesity Effects of *Capparis spinosa and Chamaemelum nobile* Aqueous Extracts in HFD Mice. *Am J Pharm Toxicol* **2**: 106–110.

Lumeng CN, Bodzin JL and Saltiel AR. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**: 175–184.

Mahmood N D, Nasir N LM, Rofiee M S, Tohid S F M, Ching S M, Teh L K, Salleh M Z, Zakaria ZA. (2014). Muntingia calabura: A review of its traditional uses, chemical properties, and pharmacological observations. *Pharm Biol* **52**: 1598–1623.

Ou B, Hampsch-Woodill M, Flanagan J, Deemer E., Prior RL, Huang D. (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J Agri Food Chem.* **50**: 2772–2777.

Pereira GA, Arruda HS, de Morais DR, Eberlin MN, Pastore GM. (2018). Carbohydrates, volatile and phenolic compounds composition, and antioxidant activity of calabura (*Muntingia calabura L.*) fruit. *Food Res Int* **108**: 264–273.

Poudel B, Nepali S, Xin M, Ki HH, Kim YH, Kim DK, Lee YM. (2015). Flavonoids from Triticum aestivum inhibit adipogenesis in 3T3-L1 cells by up regulating the insig pathway. *Mol Med Rep* **12**: 3139–3145.

Prior R, Wu X, Schaich K. (2005). Standardized methods for the determination of antioxidant capacity and phenolic in foods and dietary supplements. *J Agric Food Chem* **53:** 4290–4302.

Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. (2001). Distinct transcriptional profiles of adipogenesis *in vivo* and *in vitro*. *J Biol Chem* **276**: 34167–34174.

Sowndhararajan K, Kang SC. (2013). Free radical scavenging activity from different extracts of leaves of *Bauhinia vahlii* Wight & Arn. *Saudi J Biol Sci* **20**: 319–325.