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Original Research Article

Anti-melanogenic effect of *Amaranthus tricolor* extract on UV radiation-exposed melanoma cells

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Abstract

Purpose: *To determine the inhibitory effect of A. tricolor extract (ATE) on melanogenesis in UV radiation-exposed B16F10 cells.*
Methods: *Total phenolic and anthocyanin contents of ATE were examined by Folin-Ciocalteu and pH differential methods, respectively. The tyrosinase inhibitory effect of ATE was investigated using L-DOPA as a substrate and the suppressive effect of ATE on melanin production was determined in melanoma cells. Furthermore, the antioxidant activity was assessed by DPPH method and reactive oxygen species formation while protein expression levels were evaluated by western blot method.*
Results: *The total phenolic and anthocyanin contents were identified up to 35 mg/g extract and 0.85 mg/g extract, respectively. ATE was found to inhibit both mushroom and cellular tyrosinase at IC_{50} values of 242.2 ± 9.5 µg/mL and 202.9 ± 11.6 µg/mL, respectively. Moreover, ATE treatment reduced melanin production up to (30 ± 5) % and suppressed p38 MAPK phosphorylation at the concentration of 400 µg/mL. In addition, ATE significantly scavenged DPPH radical with EC_{50} value of 189.2 ± 8.9 µg/mL and abolished ROS formation in the cells.*
Conclusion: *These results indicate that A. tricolor extract has a potential protective effect against melanin production, suggesting its potential value in skin-lightening formulations.*

Keywords: *A. tricolor, Tyrosinase, Melanin, Melanoma cells, ROS*

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INTRODUCTION

The human skin acts as a protective barrier against physical, chemical and biological elements from the external environment. However, extensive exposure to ultraviolet (UV) radiation can harm skin cells, leading to photoaging and skin cancers. UV radiation has been recognized to be responsible for the increase in melanin synthesis via the regulation of tyrosinase activity in melanocytes and/or melanoma cells [1]. The excessive production of melanin may have biological toxicity and mutagenicity, which is likely to elevate the risk of malignant melanoma. Presently, consumers are placing significant emphasis on achieving beautiful and healthy skin. Thus, studies for the discovery of phytochemicals that are safe and effective in the prevention of UV radiation-

induced skin damages are necessary. *Amaranthus tricolor* L. is a common vegetable belonging to the family Amaranthaceae, which possess high nutritional value and delicious flavor. The pharmaceutical characteristics *of A. tricolor* have been identified through its antimicrobial, antioxidant, antitumor, anti-inflammatory, hepatoprotective, antihyperglycemic and gastric antisecretory activities [2]. Notably, various phytochemicals such as vitamins C, beta carotene, glycosides, phenolic acids, flavonoids, amaranthine, tannins and pigment compounds have been found from *A. tricolor* extract [3]. The high content of such metabolites makes *A. tricolor* promising for use in cosmetics. However, the role of *A. tricolor* on cosmetic activities such as skin whitening or UV protection has not been reported. The present study, therefore, was proposed to evaluate the beneficial effect of *A. tricolor* extract against UV-induced hyperpigmentation in melanoma cells.

EXPERIMENTAL

Materials

Amaranthus tricolor sample was purchased from Greengrocer in district 7, Ho Chi Minh City, Vietnam. Fetal bovine serum, DMEM medium, and antibiotics were purchased from Invitrogen Co. (Carlsbad, CA, USA), B16F10 cells was donated by Marine Science Institute, Jeju National University, Korea. The antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extract preparation

The dried *A. tricolor* powder was mixed with the distilled water (acidified with 0.01 % HCl) at a ratio of 1:4 (w/v), and maintained at 60 ^{o}C for 4 h. The aqueous extract was well-mixed with 2 volumes of chloroform to remove nonpolar compounds. The mixture was maintained until a clear partition between the two phases was obtained. The aqueous phase was collected and then concentrated by lyophilization to achieve a power extract with a humidity of less than 10 %. The lyophilized extract was stored at - 20 ^{o}C until used. An expected concentration was achieved by dissolving the extract into the distilled water containing 0.01 % HCl.

Determination of anthocyanin content

The anthocyanin content from *A. tricolor* extract (ATE) was identified via the pH differential assay according to Lee and colleagues [4]. The KCl buffer (0.025 M, pH = 1) and CH_3COOH buffer (0.4 M, pH = 4.5) were used to dissolve the extract and the absorbance was read at 520 and 700 nm for both solutions. The total anthocyanin was considered to be cyanidin-3-glucoside equivalent.

Total phenolic content (TPC) analysis

The total phenolic content in the extract was determined according to the Folin-Cocialteau method [5]. The value was extrapolated from a standard curve of gallic acid and expressed as gallic acid equivalent per gram extract.

Tyrosinase inhibitory assay

Tyrosinase inhibitory assay were performed according to the method of Nguyen and colleagues [6]. A mixture containing 80 µL of phosphate buffer (pH 6.8, 50 mM), 10 µL of the extract at different concentrations and 30 µL of mushroom tyrosinase solution (100 IU/mL, diluted in phosphate buffer) was incubated in 96 well-plate for 15 min at 25 ^{o}C. Subsequently, 80 µL of L-DOPA solution (10 mM, diluted in phosphate buffer) was added to the mixture and the reaction was maintained for 10 min at 25 ^{o}C. The orange dopachrome production from colorless L-DOPA was measured through absorbance at 490 nm by a microplate reader (BioTek Instruments, USA). Kojic acid was used as a positive control. Each measurement was made in triplicates.

The inhibitory activity (I) of the extract on enzyme activity was calculated using Eq 1.

$$I\ (\%) = ((A_0 - A_1)/A_0)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

where A_0 = + Enzyme + L-DOPA-Extract; A_1 = + Enzyme + L-DOPA + Extract

Cell viability

The effect of the extract on B16F10 cells (donated by Marine Science Institute, Jeju National University, Korea) was examined by MTT assay [7]. The cell viability was compared against the control group.

Total melanin content assay

Melanoma (B16F10) cells were pretreated with different concentrations of the extract or Kojic acid before exposure to UV radiation at a density of 100 mJ/cm^2 for 5 min (twice a day). The cells were maintained in a 5 % CO_2 incubator at 37 ^{o}C for 24 h. Melanin content in the melanoma cells was examined according to existing protocol [6].

The UV exposed-group without extract or Kojic acid treatment was set at 100 % melanin content (Control group). The melanin content of the study group that was pretreated with the extract or Kojic acid before UV radiation exposure was expressed as relative (%) to the control group.

Cellular tyrosinase inhibitory assay

For the cellular tyrosinase assay, B16F10 cells were pretreated with different concentrations of the extract or Kojic acid before exposure to UV radiation at a density of 100 mJ/cm^2 for 5 min (twice a day). The assay was done according to existing protocol [6]. Each measurement was made in triplicates. The inhibitory activity (I) of the extract on cellular enzyme activity was calculated using Eq 1. In this case however, A_0 represent control group, while A_1 is for study group.

DPPH (2,2-diphenyl-1-picryl hydrazyl) assay

This assay was performed to determine the antioxidant capacity of the natural product. In this assay, 100 µL of the extract at the concentration of 50 - 400 µg/mL was mixed with 100 µL of DPPH (3 mM). The mixture was pipetted and subsequently maintained in a dark room for 30 min. The absorbance of the mixtures was then measured at 490 nm using a microplate reader. Vitamin C was used as positive control.

Reactive oxygen species assay

The effect of the extract on reactive oxygen species (ROS) production from B16F10 cells was examined. The cells were pretreated with the extract at different concentrations for 24 h. The cells were then incubated with 100 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min in the dark at 37 ^{o}C before being exposed to UV radiation (100 mJ/cm^2) for 5 min. Finally, the fluorescence intensity was visualized under an inverted fluorescence microscope (Oxion, Euromex, Netherlands).

Western blot analysis

Melanoma (B16F10) cells were pre-treated with the extract at a concentration range of 100 – 400 µg/mL for 24 h. The cells were then exposed to UV radiation at a density of 100 mJ/cm^2 for 5 min. The total protein was extracted by RIPA lysis buffer. The targeted proteins were detected using proper antibodies according the protocol reported by the previous study [8]. The relative band intensity was measured by ImageJ software.

Statistical analysis

Values are presented as mean ± standard deviation (SD). The data was analyzed using the one-way ANOVA test in the Statistical Package for the Social Sciences (SPSS). Statistical significance among groups was considered at $p < 0.05$.

RESULTS

Total phenolic and anthocyanin contents of *A. tricolor* extract (ATE)

The result of total phenolic content was based on a calibration curve ($y = 0.0107x + 0.019$, $R^2 = 0.9964$) established using gallic acid (0 – 100 µg/mL). The total phenolic content of *A. tricolor* extract was determined up to 35 mg gallic acid equivalent/g extract. Moreover, the total anthocyanin content from *A. tricolor* extract was identified up to 0.85 mg cyanidin-3-glucoside equivalent/g extract.

The inhibition of ATE on tyrosinase activity

The tyrosinase inhibitory activity of ATE and Kojic acid is shown in Table 1. The inhibitory effect on tyrosinase activity varied among different ATE and Kojic acid concentrations. The results indicate that both ATE and Kojic acid have a direct and indirect inhibitory activity against mushroom and cellular tyrosinase, respectively. The extract (ATE) inhibited mushroom and cellular tyrosinase activities at IC_{50} values of 242.2 ± 9.5 µg/mL and 202.9 ± 11.6 µg/mL, respectively. Meanwhile, kọjic acid inhibited mushroom and cellular tyrosinase activity at IC_{50} values of 88.4 ± 1.4 µg/mL and 106.7 ± 13.5 µg/mL, respectively. From the result, the inhibitory effect of ATE on cellular tyrosinase was higher than that of mushroom tyrosinase. However, the inhibition of ATE on tyrosinase activity was significantly weaker compared to that of Kojic acid ($p < 0.05$).

Effects of ATE on the melanin production

According to the results shown in Figure 1, ATE and Kojic acid reduced melanin production in a dose-dependent manner. The extract (ATE) and Kojic acid significantly decreased the melanin production from UV radiation-exposed B16F10 cells at the highest concentration treatment, compared with that of the control. Melanin content was decreased up to 30 ± 5 and 55 ± 2 % by 400 µg/mL of ATE and 200 µg/mL of Kojic acid, respectively. The inhibitory effect of ATE and Kojic acid was not observed due to the

cytotoxic effect at the concentration range of 100 - 600 and 50 - 400 µg/mL, respectively.

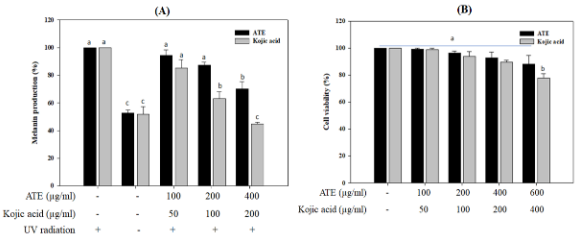

Figure 1: The inhibition (A) and cytotoxic effect (B) of ATE and Kojic acid on melanin production from UV radiation-exposed B16F10 cells. The different letters indicate the significant difference among group at $p < 0.05$

Antioxidant activity of ATE

The results showed that both ATE and Kojic acid were able to scavenge DPPH radical in a concentration-dependent manner (Table 2). The DPPH scavenging activities of ATE and Kojic acid were found at up to 76.3 and 64.1 % at the concentration of 400 µg/mL, respectively. Moreover, the half-maximal effective concentrations (EC_{50}) of ATE and Kojic acid on DPPH scavenging activity were shown at values of 189.2 ± 8.9 µg/mL and 259.1 ± 10.2 µg/mL, respectively. The DPPH scavenging activity of ATE was found to be significantly higher than that of Kojic acid. In addition, the inhibitory effect of ATE and Kojic acid on cellular ROS production in B16F10 cells was further assessed. It was observed that exposure of B16F10 cells to UV radiation remarkably increased cellular ROS intensity which was indicated by an increase in the intense green fluorescence of DCF. However, the treatment of ATE or Kojic acid at the concentration of 400 µg/mL effectively decreased the intensity of green fluorescence, indicating the suppressive effect of ATE and Kojic acid on the UV radiation-induced ROS production in the melanoma cells (Figure 2). Similarly, the inhibitory activity of ATE on cellular ROS production was higher than that of Kojic acid at the same concentration treatment.

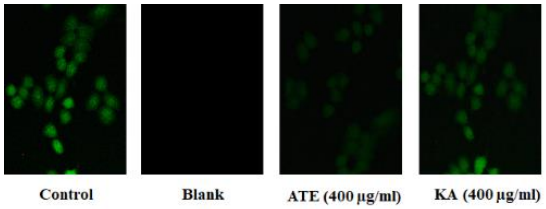

Figure 2: The inhibitory activity of ATE and Kojic acid on ROS production from UV radiation-exposed B16F10 cells. The fluorescence intensity was visualized under an inverted fluorescence microscope

Effect of ATE on MAPKs signaling pathway

In order to investigate the involvement of MAPKs on the ATE-induced suppression of melanogenesis, the phosphorylated levels of p38, ERK and JNK were examined by Western blot analysis. As shown in Figure 3, UV radiation-exposed melanoma cells induced a high level of p38, ERK and JNK phosphorylation. Conversely, ATE treatment significantly reduced the phosphorylation levels of p38, but not ERK and JNK at the concentration of 400 µg/mL. These results suggest that ATE inhibited melanin production by downregulating ERK phosphorylation in the MAPKs signaling pathway.

DISCUSSION

Plant extracts have shown promising potential in providing UV protection for the skin. Many plants produce secondary metabolites, such as flavonoids, phenolic compounds and carotenoids, which possess antioxidant and photoprotective properties.

Table 1: Inhibitory effect of ATE or Kojic acid on mushroom and cellular tyrosinase activity

Sample	Concentration range (µg/mL)	Model	Regression equation	IC_{50} (µg/mL)
ATE	10-400	Mushroom tyrosinase	y=0.1651x+10.004 (R^2=0.9014)	242.2±9.5
	100-400	Cellular tyrosinase	y=0.1843x+12.596 (R^2=0.9155)	202.9±11.6
Kojic acid	40-120	Mushroom tyrosinase	y=0.6857x-10.637 (R^2=0.9263)	88.4±1.3
	50-200	Cellular tyrosinase	y=0.5557x-9.3061 (R^2=0.9133)	106.7±13.5

Table 2: DPPH scavenging effect of ATE and Kojic acid

Sample	Concentration range (µg/mL)	Regression equation	IC_{50} (µg/mL)
ATE	50-400	y=0.1306x+27.425 (R^2=0.8913)	189.2±8.9
Kojic acid		y=0.1129x+20.75 (R^2=0.9631)	259.1±10.2

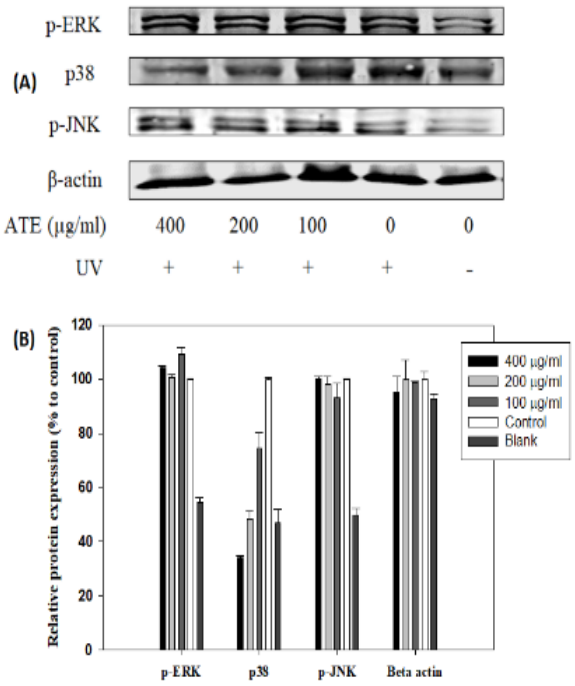

Figure 3: The regulation of ATE on MAPKs signaling pathway in UV radiation-exposed B16F10 cells. (A) Representative blot showing the target proteins. (B) The relative band intensity

In this study, the high levels of the total phenolic and anthocyanin content from ATE may be considered as protective components against UV radiation exposure. Tyrosinase plays a pivotal role in melanogenesis, the process by which melanin pigment is synthesized. Factors such as hormonal signals, UV exposure and genetic variations can influence the expression and activity of tyrosinase. Herein, the role of ATE on tyrosinase inhibition has been determined by an *in vitro* model. The extract has been shown to be effective in the inhibition of both mushroom and cellular tyrosinase activities. It was reported that polyphenols, phenolic acids and anthocyanin possess an inhibitory effect on tyrosinase activity. They exert their inhibitory effects on tyrosinase through competitive or non-competitive inhibition, chelation of copper ions and antioxidant activity [9]. Therefore, the presence of phenolic and anthocyanin compounds in ATE may contribute to the inhibition of tyrosinase activity, thus reducing melanin production in melanoma cells. The inhibitory activity of ATE on tyrosinase activity is stronger than that of *A. lividus* L. (IC_{50} = 2.27 mg/ml) [10], but weaker than *Perilla frutescens* (IC_{50} = 140 µg/mL) [6]. These results indicate the potential inhibitory effect of ATE on tyrosinase activity which contribute to the suppression of melanin synthesis from the melanoma cells.

Previous studies have established that UV radiation from the sun can lead to the production ROS in the skin. The generated ROS activates signaling pathways that lead to the upregulation of genes involved in melanin production, including genes encoding tyrosinase, TRP-1 and TRP-2. Increased expression of these genes result in higher levels of melanin production in melanocyte [11]. Therefore, the antioxidants may help to suppress the melanin production by scavenging free radicals and intracellular ROS. In this sense, the role of ATE as an effective antioxidant is buttressed by its DPPH radical scavenging activity and suppressing of ROS production in UV-exposed B16F10 cells. According to Fu and colleagues, the intervention of antioxidants slows down the rate of melanin formation [12]. Moreover, L-glutathione, an important antioxidant naturally produced in the body, exhibited inhibitory activity against UVB-induced melanogenesis [13]. As seen from the results, the antioxidant property of ATE partly contributed to the suppression of tyrosinase genes, leading to reduction of melanin production in melanoma cells.

Mitogen activated protein kinases (MAPKs) play a crucial role in coordinating melanin production and determining pigmentation levels in the skin [14]. Earlier research demonstrated that the activation of JNK and ERK resulted in the suppression of melanin synthesis [15]. Meanwhile, the activation of MITF through p38 MAPK phosphorylation serves as a stimulus for melanogenesis, leading to a production of melanin [16]. In the present study, ATE treatment causes a significant suppression on p38 MAPK phosphorylation in B16F10 cells. Choi and colleagues also showed that SB203580, a specific inhibitor of p38 MAPK, suppressed the MITF expression, leading to decrease in melanin production in B16F10 cells [15]. These results indicate that the inhibition of tyrosinase activity and melanin production by ATE may be related to the downregulation of p38 MAPK phosphorylation in melanoma cells.

CONCLUSION

Amaranthus tricolor extract (ATE) suppresses melanogenesis in UV radiation-exposed melanoma cells. Its inhibitory effect on melanogenesis is evidenced by blocking tyrosinase activity, reducing melanin production, scavenging free radicals and inactivating p38 MAPK phosphorylation. Exploiting the tyrosinase inhibitory properties of *A. tricolor* extract holds promise in developing effective strategies for managing hyperpigmentation disorders, skin-lightening formulations and other related

applications in the skincare and cosmetic industries.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities relating to claims relating to the content of this article will be borne by the authors. Dai-Hung Ngo designed and conducted the study; Young-Sang Kim, Thanh Sang Vo, and Dai-Hung Ngo supervised data collection, analyzed and interpreted the data; Dai-Hung Ngo and Dai-Nghiep Ngo prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

REFERENCES

1. *Gilchrest BA, Park HY, Eller MS, Yaar M. Mechanisms of ultraviolet light-induced pigmentation. Photochem Photobiol 1996; 63(1): 1-10.*
2. *Srivastava R. An updated review on phyto-pharmacological and pharmacognostical profile of Amaranthus tricolor: A herb of nutraceutical potentials. Pharma Innov J 2017; 6(6):124-129.*
3. *Jahan F, Bhuiyan MNH, Islam MJ, Ahmed S, Hasan MS, Bashera MA, Waliullah M, Chowdhury AN, Islam MB, Saha BK, et al. Amaranthus tricolor (red amaranth), an indigenous source of nutrients, minerals, amino acids, phytochemicals, and assessment of its antibacterial activity. J Agric Food Res 2022; 10: 1-7.*
4. *Lee J, Durst RW, Wrolstad RE. Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study. J AOAC Int 2005; 88(5): 1269-1278.*
5. *Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am J Enol Vitic 1965; 16(3): 144-158.*
6. *Do T-H, Huynh N-TJJoHP. In vitro anti-melanogenic effect of Perilla frutescens leaf extracts. J Herbmed Pharmacol 2021;11(1):48-54.*
7. *Mazloum-Ardakani M, Barazesh B, Moshtaghioun SM, Sheikhha MH. Designing and optimization of an electrochemical substitute for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. Sci Rep 2019; 9(1): 1-8.*
8. *Vo T-S, Kong C-S, Kim S-K. Inhibitory effects of chitooligosaccharides on degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells. Carbohydr. Polym 2011; 84(1): 649-655.*
9. *Zolghadri S, Bahrami A, Hassan Khan MT, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, Saboury AA. A comprehensive review on tyrosinase inhibitors. J Enzyme Inhib Med Chem 2019; 34(1): 279-309.*
10. *Çelikok GH, Özden TY, Saçan Ö, Can A, Yanardağ R. In vitro inhibitory potential of Amaranthus lividus L. against therapeutic target enzyme. İstanbul J Pharm 2022; 52(1):73-79.*
11. *Kamiński K, Kazimierczak U, Kolenda T. Oxidative stress in melanogenesis and melanoma development. Contemp Oncol (Pozn) 2022; 26(1): 1-7.*
12. *Fu W, Wu Z, Zheng R, Yin N, Han F, Zhao Z, Dai M, Han D, Wang W, Niu L. Inhibition mechanism of melanin formation based on antioxidant scavenging of reactive oxygen species. Analyst 2022; 147(12): 2703-2711.*
13. *Nagapan TS, Lim WN, Basri DF, Ghazali AR. Oral supplementation of L-glutathione prevents ultraviolet B-induced melanogenesis and oxidative stress in BALB/c mice. Exp Anim 2019; 68(4): 541-548.*
14. *D'Mello SA, Finlay GJ, Baguley BC, Askarian-Amiri ME. Signaling Pathways in Melanogenesis. Int J Mol Sci 2016; 17(7): 1-18.*

15. Choi H, Yoon J-H, Youn K, Jun M. Decursin prevents melanogenesis by suppressing MITF expression through the regulation of PKA/CREB, MAPKs, and PI3K/Akt/GSK-3β cascades. Biomed. Pharmacother 2022; 147: 1-15.

16. Jung E, Kim JH, Kim MO, Jang S, Kang M, Oh SW, Nho YH, Kang SH, Kim MH, Park SH, et al. Afzelin positively regulates melanogenesis through the p38 MAPK pathway. Chem Biol Interact 2016; 254:167-172.