In vitro Antioxidant Activities and Cytotoxicity of Peanut Callus Extract

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Abstract

Resveratrol is widely reported to be beneficial to health by possessing antioxidative, anti-inflammatory, anti-carcinogenic, and antitumor properties. In this study, peanut callus was induced on agar solidified Murashige and Skoog medium supplementing with 2 mg/mL of 2,4-dichlorophenoxyacetic acid, 1 mg/mL of 1-naphthaleneacetic acid, and 1 mg/mL of 6-benzylaminopurine. Callus was initially formed from seed as compact yellow texture after 4 weeks with a high yield (90.24%) that was calculated based on the initial weight of seed. The amount of resveratrol was increased by callus culture (190.11 ppm) in comparison to peanut seed (not detected). To investigate the effect of solvent on total phenolic content, antioxidant, and anti-tyrosinase activities, callus was extracted with various concentrations of ethanol (0, 25, 50, 70 and 95 %v/v). The highest phenolic content and bioactivities were found in 95%v/v ethanol extract. Ethanolic peanut callus extract showed the highest phenolic content (0.42 mg gallic acid equivalents per ml extract), ferric reducing power (0.55 mg ascorbic acid equivalents per ml extract), DPPH radical scavenging (88.78%), and tyrosinase inhibition (79.89%). In vitro cytotoxicity of the extracts was tested on human keratinocyte cells by MTT assay. Results showed that peanut callus extract (50% cytotoxic concentration; CC_{50} 16.3% v/v) was found to be lower toxic to keratinocytes than peanut seed extract (CC_{50} 10.57%v/v). Thus, induction of callus of peanut enhances resveratrol, phenolic compounds, antioxidant and anti-tyrosinase activities which could find interesting applications in food, dietary supplement and cosmetic products.

Keywords: Antioxidant, Callus, Cytotoxicity, Peanut, Resveratrol

1. Introduction

Peanut (*Arachis hypogaea* L), belonging to the Leguminosae family, is an important food crop and agriculture economy of Thailand. Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is the major compound in peanut that belongs to the class of phenyl-propanoid compounds known as stilbenoids or stilbenes. This compound has been shown to confer protection to the peanut plant in countering pathogens and therefore are believed to function as phytoalexins (Condori *et al.*, 2010). Resveratrol is interesting compounds to use as anti-aging skin agent due to it is a high potent compound on antioxidant, anti-inflammatory, and anti-infective properties (Baxter, 2008).

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Received: 5 September 2018/ Revised: 8 October 2018/ Accepted: 3 March 2019

Over the last few years, the utilization of plant cell culture technology are increase of interesting for harvesting plant metabolites which are independent in seasonal and environmental restraints (Schmid et al., 2008). This technique is based on propagation of plant callus or stem cells which contains many compounds contributing to a beneficial protecting effect of skin cells under sterile conditions (Schürch et al., 2008). There has been reported that resveratrol was 2–20 times increased by plant callus culture technique (Hasan and Bae, 2017).

The present study is aimed to determine the produced resveratrol in peanut callus culture and evaluate their total phenolic content, ferric reducing power, DPPH radical scavenging, and anti-tyrosinase activities. Moreover, cytotoxicity of extracts was estimated on human keratinocyte cells by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to estimate the safety and dosage for cosmetic usage.

2. Materials and Methods

2.1 Peanut callus induction

Peanut seeds were sterilized with 40% v/v Clorox® for 10 min and rinsed with autoclaved distilled water. Then, the sterilized seed was inoculated on agar solidified basal Murashige and Skoog medium supplemented with 30 g/L of sugar, 2 g/L of gellant gum, 2 mg/mL of 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/mL of 1-naphthaleneacetic acid (NAA), and 1 mg/mL of 6-benzylaminopurine (BAP). Then, cultured seed was incubated in the controlled temperature conditions (25 ± 2 °C) for 4 weeks and harvested the callus formation.

2.2 Analysis of resveratrol

The resveratrol content in sample was analyzed by reversed phase high-performance liquid chromatography (HPLC) (Song *et al.*, 2009). The extracts were analyzed by using Poroshell 120 EC-C18 (250 × 4.6 mm, 4 µm) with Poroshell 120 guard column (5 × 4.6 mm, 4 um). Samples were separated at 40 °C and 1 mL/min of flow rate. Detection wavelength was 320 nm. The elution program was performed as mixture of 40% v/v of methanol and 60% v/v of water for 30 min.

2.3 Extract preparation

A portion (1 g) of samples (peanut seed and callus) were extracted with 10 mL of various concentration of ethanol including 0, 25, 50, 75 and 95 % v/v. After 24 h, the extracted solutions were filtered with Whatman® no. 1 filter paper and stored at 4 °C until analysis. The effect of ethanol concentration as extracting solvents was evaluated on total phenolic content, antioxidant and anti-tyrosinase activities.

2.4 Determination of total phenolic content

The total phenolic content (TPC) was analyzed using the Folin-Ciocalteu assay (Vichit and Saewan, 2015). Briefly, the extracts (20 µL) were mixed with 50 µL of deionized water, 20 µL of Folin-Ciocalteu reagent and 125 µL of 7% w/v sodium carbonate. Samples were incubated for 90 min at room temperature. The absorbance was measured at 750 nm using a microplate reader (Biochrom, USA). TPC of samples was expressed as gallic acid equivalents (mg GAE/mL extract) based on standard curve of gallic acid.

2.5 Determination of Ferric reducing power

Ferric reducing power (FRAP) was determined according to Vichit and Saewan (2015). The extracts (25 µL) were mixed with 50 µL of 1% w/v potassium ferricyanide and stood for 60 min at room temperature. Trichloroacetic acid (25 µL) and deionized water (75 µL) were added and the absorbance was measured at 700 nm using the microplate reader as absorbance 1 (A1). Then, 25 µL of 0.1 % w/v ferric chloride was added and the absorbance measured at 700 nm again as absorbance 2 (A2). The optical density of sample was calculated using the following equation:

$$\text{Optical density} = (A2 - A1)_{sample} - (A2 - A1)_{control}$$

The reducing power activity was evaluated using the ascorbic acids calibration curve. The results were expressed as ascorbic acids equivalent (mg AAE/ml extract).

2.6 Determination of DPPH radical scavenging activity

Scavenging activity of DPPH free radicals was determined according to Vichit and Saewan (2015). The extract (5 µL) was added 195 µl of 0.1 mM of DPPH solution and incubated at room temperature for 30 min. Absorbance was measured at 515 nm using the microplate reader. The scavenging activity was calculated as follows:

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

2.7 Determination of Anti-tyrosinase activity

Tyrosinase inhibitory was described by Saewan *et al.* (2011), using a modified dopachrome method with L-DOPA as a substrate. The extracts (20 µl) were added to the mixture of 0.1 M phosphate buffer (pH 6.8; 100 µL) and 1 mM L-DOPA (40 µl). The samples were incubated at 37 °C for 10 min. Then, 40 µL of 200 unit/mL tyrosinase in 0.1 M phosphate buffer (pH 6.5) was added to the sample and incubated for 15 min at 37 °C. Absorbance was measured at 475 nm. The percentage of tyrosinase inhibition was calculated as:

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

2.8 Cytotoxicity

The extracts were investigated cytotoxicity on Human primary epidermal keratinocyte cells. The cytotoxicity assessment was performed by MTT assay (Vichit and Saewan, 2015; Vichit and Saewan, 2016). Cells were placed in wells of a 96-well plate and treated with diluted extracts at concentrations of 0–20% v/v for 24 h. Afterward, the culture medium was removed. The 50 µL of 0.1 mg/mL of MTT solution was added to each well and incubated for 4 h. DMSO (100 µL) was added to all wells and incubated for 30 min at room temperature. The absorbance of each sample was measured at 570 nm using the microplate reader. The percentage of cell viability was calculated using the formula,

$$\text{Viable cell (\%)} = (A_{\text{treated group}} / A_{\text{untreated group}}) \times 100$$

The cytotoxic activity was calculated and expressed as the 50 % cytotoxic concentration (CC_{50}) of extract.

2.9 Statistical analysis

All measurements were performed in triplicate. The obtained data were statistically analyzed using the SPSS program version 11.5 for window (SPSS Inc, Chicago, IL, USA) and the differences were considered significant when $p < 0.05$. The comparison of data between each extracting solvent was analyzed by using One Way Analysis of Variance (ANOVA) with Duncan's multiple range test. The comparison of data between seed and callus extract was analyzed by using independent sample t-test.

3. Results and Discussion

3.1 Callus induction

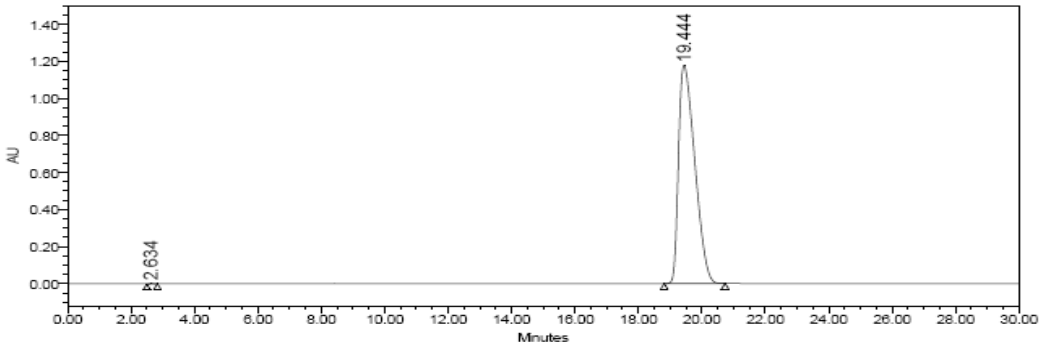
Peanut callus was successfully induced from the seed after induction on the callus medium for 4 weeks (Figure 1). The seed provided high yield of callus (90.24 ± 4.98%) as yellow color compact texture.

Figure 1 Appearance of peanut seed and callus

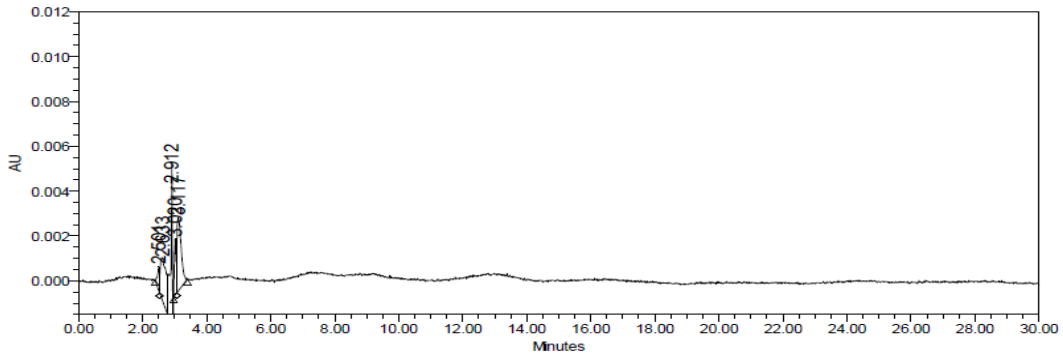
3.2 Resveratrol content

The ethanolic seed and callus extract was evaluated resveratrol content by using HPLC. The chromatogram of the resveratrol standard and samples are shown in Figure 2. Resveratrol did not detect in peanut seed extract. Wang and Pittman (2008) reported that resveratrol content in peanut seeds was depend on germplasm which was on average about 0.5 ppm and a statistically significant variation (from 0.125 to 1.626 ppm, at least a ten-fold difference). Moreover, Kisbenedek *et al*. (2014) indicated that the level of resveratrol is minor in non-roasted peanut seed. Thus, the peanut seed using in this study maybe contains very low resveratrol content until cannot detected in extract. However, peanut seed extract showed many unknown substance peak (Figure 2b) that maybe contain other compounds in seed extract and cannot identified with this HPLC condition. While, resveratrol in peanut callus extract was found 190.11 ± 2.63 ppm. This result showed that resveratrol content in peanut could be induced by callus culture technique.

(a)



(b)



(c)

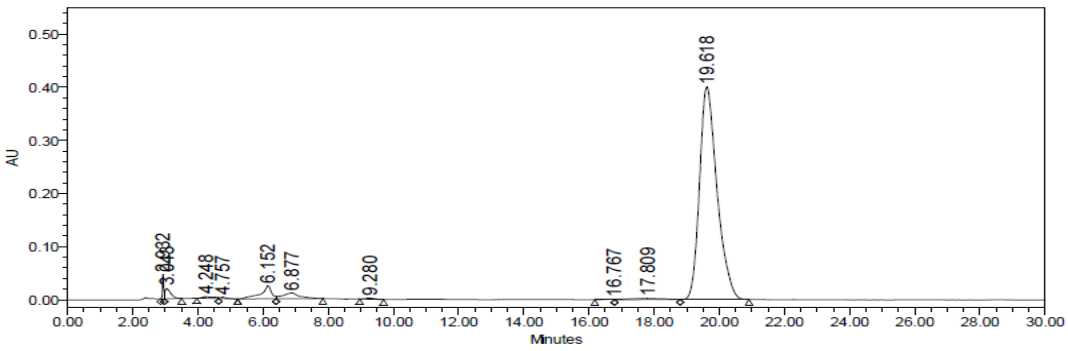


Figure 2 HPLC chromatogram of **(**a**)** resveratrol standard, **(**b**)** seed extract, and **(**c**)** callus extract**.**

3.3 Extracting solvent concentrations and bioactivities

To select the best extracting solvent, callus was extracted with various concentration of ethanol (0, 25, 50, 75, and 95 %v/v) and evaluated for their total phenolic content, antioxidant, and anti-tyrosinase activities. The appearance of seed and callus extracts was shown in Figure 3.

Phenolic compounds act as antioxidant through various mechanisms like free radicals scavenging, transition metal chelation and lipid peroxidation inhibition (Sruthi and John Zachariah, 2017). Thus, phenolics became an interesting compound for health benefit and anti-aging activities. In this study, total phenolic content of extracts was determined using the Folin-Ciocalteu assay (Figure 4a). Total phenolic content of seed extract was in the range of 0.07–0.14 mg GAE/ml extract, while, callus extract was in the range of 0.35–0.42 mg GAE/mL extract. Furthermore, the content of phenolic compounds in peanut seed and callus extracts were related with resveratrol content that detected by HPLC due to resveratrol is a natural phenolic compound.

Antioxidant activities were determined by 2 assays including ferric reducing power (FRAP) and DPPH radical scavenging (DPPH) as shown in Figure 4b and 4c, respectively. Callus extracts (0.49–0.55 mg AAE/ml extract) exhibited high effectiveness in FRAP with 4.5 times when comparison to peanut seed extracts (0.10–0.13 mg AAE/mL extract). The DPPH scavenging of callus extract (61.74–88.78%) indicated strong hydrogen-donating ability that was significantly higher activity than seed (18.26–47.83%).

Tyrosinase, a copper-containing enzyme, is a key enzyme in melanin biosynthesis, involved in determining the color of mammalian skin and hair (Parvez *et al*., 2007). Tyrosinase inhibitors are increasingly used in the cosmetic industry for anti-pigmenting effects. In this study, the anti-tyrosinase activity of extracts was determined by using a modified dopachrome method with L-DOPA as the substrate (Figure 4d). Peanut callus extract (79.89–51.51%) showed about 2.6 times more anti-tyrosinase activity than seed extract (14.47–35.81%).

For callus extraction, the antioxidant and anti-tyrosinase activities were increase with increasing of the ethanol concentration. The highest ethanol concentration (95%v/v) showed significantly maximum phenolic content and bioactivities than the lower concentrations of ethanol. There is possible the compound in callus can easily dissolve in high concentration of ethanol. This may be due to the fact that aqueous solvents are suitable for extracting some bioactive compounds with strong polarity; ethanol or ethanol/water solvent is suitable for extracting some bioactive compounds with broad range of polarity (Sun *et al*., 2015). Usually, plant callus contains a complex matrix of ingredients like salts, acids, phenols, sugars, lipids, proteins, and other undefined fractions of compounds (Schürch *et al*., 2008). Thus, these

results imply that 95 %v/v ethanol solvent may be appropriate to extract peanut callus. While, 50%v/v ethanol provide better bioactivities than others concentration in peanut seed. There is possible the compound in peanut seed is high polarity compounds that easily dissolve in 50%v/v ethanol/water and demonstrated high antioxidant activity both FRAP and DPPH.

Moreover, several studies suggested that callus formation enhance the antioxidative secondary metabolites production and increase bioactivities (Abouzid *et al*., 2010; Ali and Elnour, 2014; Bolda *et al*., 2011; Kalidass *et al*., 2010). A strong correlation between total phenolic content and bioactivities was found 0.995 for ferric reducing power, 0.927 for DPPH radical scavenging, and 0.900 for anti-tyrosinase activity. These results suggested that phenolic compounds were the major active component that affect to antioxidant and anti-tyrosinase activities in peanut seed and callus.

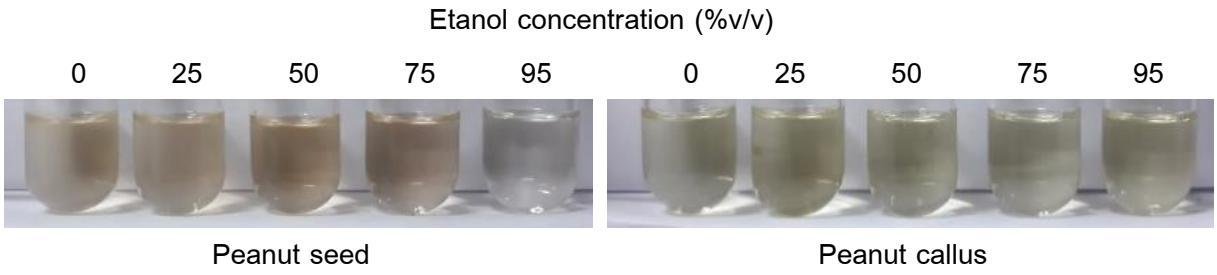


Figure 3 Appearance of peanut seed and callus extract

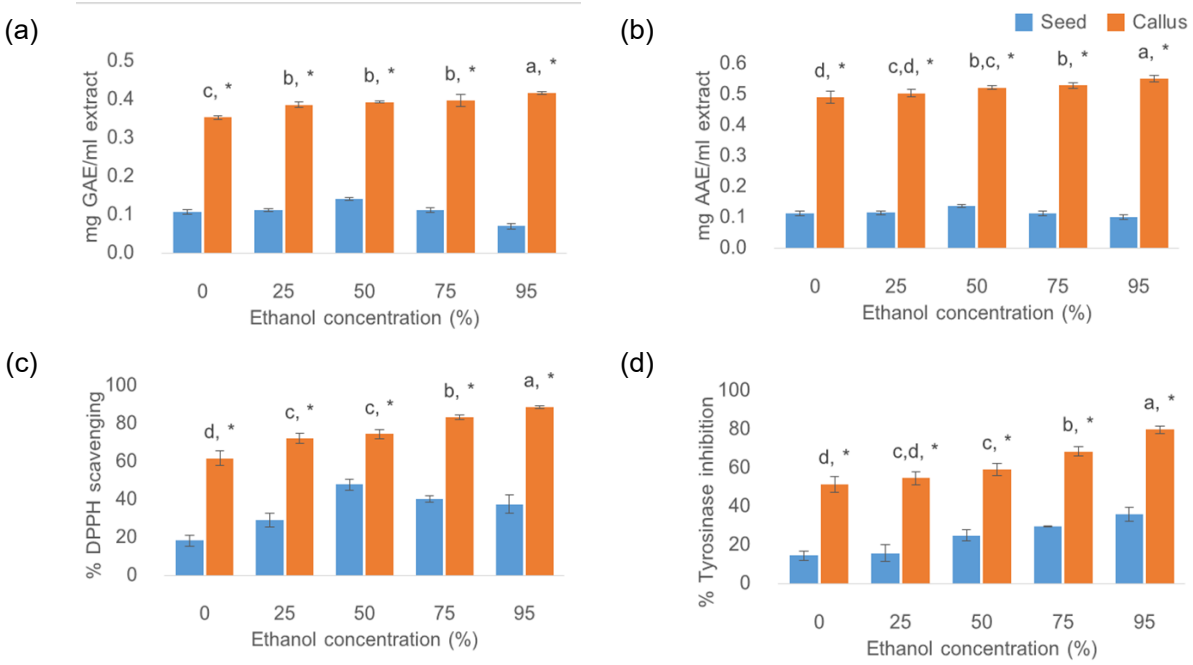


Figure 4 Effect of ethanol concentration on (a) total phenol content, (b) ferric reducing power, (c) DPPH radical scavenging, and (d) anti-tyrosinase activity. a,b,c,.. indicate significant differences (p < 0.05) of data

of various ethanol concentration. * indicate significant differences ($p < 0.05$) between seed and callus extract at same ethanol concentration

3.4 Cytotoxicity

Keratinocytes are the major cell type in the epidermis and have a critical role in the complex process of wound healing (Pastar *et al.*, 2008). In addition to activities, the potential toxicity of the extract on skin cells is an important considering issue when evaluating the possibility of plant to use in cosmetic products. Thus, the cytotoxicity of peanut seed and callus was estimated by the MTT assay using in human keratinocyte cells treated with varying concentration of extract. MTT assay is a colorimetric assay based on assessing the cell metabolic activity that depend on the conversion of yellow tetrazolium MTT to formazan by viable cells and intensity of color product is directly proportional to the cell viability number (Bahuguna *et al.*, 2017).

As shown in Figure 5, the extracts showed cytotoxicity in the dose dependent. At the lower than 5% v/v of peanut seed and 10% v/v of callus extracts displayed more than 90% cell survival. The cytotoxic activity of extracts was calculated and expressed as the $CC_{50,}$ the concentration causing 50% viable cell. The CC_{50} values of the peanut seed extract was 10.57 ± 0.46% v/v and callus extract was 16.38 ± 0.57% v/v. Thus, peanut callus extract was less cytotoxicity to skin cell than seed extract.

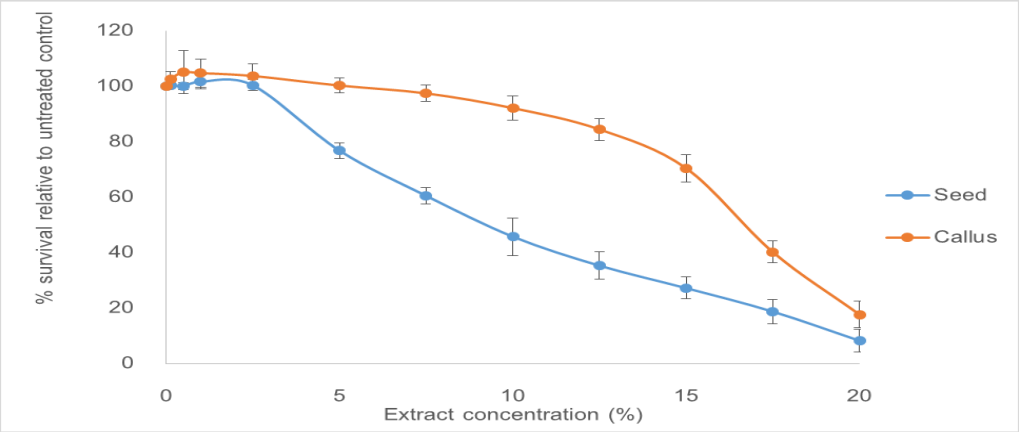


Figure 5 The percentage of cells viability after exposure to peanut seed and callus extracts

4. Conclusion

Peanut callus was successful induced on agar solidified MS medium supplementing with sugar, 2,4-D, NAA, and BAP for 4 weeks. The obtained result indicated that the callus is rich in antioxidant and anti-tyrosinase activities which can be attributed to the high resveratrol and phenolic compounds. Peanut callus extract appeared to be a low cytotoxicity on human

keratinocyte cells. Therefore, peanut callus extract have a potential to be useful ingredients in cosmetic products.

Acknowledgements

The authors express thanks to The Thailand Research Fund for financial support and to Mae Fah Luang University for providing scientific equipment and facilities for this work.

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