Research Article

สารฟินอลิกและฟลาวานอยด์และฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดเอทานอลของพรอพอลิส

ปัทมา ชัยเลิศวณิชกุล[1], ธิติมา นามสิริกุล[2], ธีระศักดิ์ ดำรงรุ่งเรือง[3], จอมใจ พีรพัฒนา[4]*

[1] รองศาสตราจารย์, ภาควิชาทันตกรรมบูรณะ คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

[2] ทันตแพทย์, โรงพยาบาลกำแพงแสน จังหวัดนครปฐม 73140

[3] รองศาสตราจารย์, ภาควิชาวินิจฉัยโรคช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

[4] รองศาสตราจารย์, ศูนย์วิจัยและพัฒนาผลิตภัณฑ์สุขภาพจากสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

***ติดต่อผู้นิพนธ์ :** คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002 โทรศัพท์ 086-862 9401
โทรสาร 043-362 092 อีเมล์ jomsuj@kku.ac.th

บทคัดย่อ

สารฟินอลิกและฟลาวานอยด์และฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดเอทานอลของพรอพอลิส

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติของสารสกัดพรอพอลิสไทย เพื่อพัฒนาเป็นวัสดุปิดทับเนื้อเยื่อในของฟันมนุษย์ โดยนำพรอพอลิสที่ขูดได้จากรังผึ้งในจังหวัดหนองคายมาสกัดด้วย 95% เอทานอล วิเคราะห์ปริมาณฟลาโวนอยด์รวมโดยวิธี Aluminum chloride colorimetric method ร่วมกับวิธี 2,4- dinitrophenylhydrazine colorimeteric method วิเคราะห์ปริมาณฟีนอลิกรวมโดยวิธี Folin-Ciocalteau colorimetric method ผลการศึกษาสารสกัดพรอพอลิสไทยมีฟลาโวนอยด์เข้มข้นร้อยละ 10 (โดยน้ำหนักของสารสกัดพรอพอลิสไทย) และฟีนอลิกเข้มข้นร้อยละ 2.95 (โดยน้ำหนักของสารสกัดพรอพอลิสไทย) สารสกัดพรอพอลิสไทย สามารถต้านเชื้อเสตร็ปโตคอคคัสมิวแทนส์ และแลคโตบาซิลลัสคาเซไอ โดยความเข้มข้นต่ำสุดที่ยับยั้งเชื้อเสตร็ปโตคอคคัสมิวแทนส์ และแลคโตบาซิลลัสคาเซไอ คือ 22.5 มิลลิกรัม/มิลลิลิตร และ 11.25 มิลลิกรัม/มิลลิลิตร ตามลำดับ และความเข้มข้นต่ำสุดที่ฆ่าเชื้อเสตร็ปโตคอคคัสมิวแทนส์ และแลคโตบาซิลลัสคาเซไอ คือ 45 มิลลิกรัม/มิลลิลิตร สารสกัดพรอพอลิสไทยความเข้มข้น 90 มิลลิกรัม/มิลลิลิตร ไม่เป็นพิษต่อเนื้อเยื่อในของฟันมนุษย์ที่เวลา 6, 12, 24 และ 72 ชั่วโมง ผลจากการศึกษานี้พบว่า สารสกัดพรอพอลิสไทยมีคุณสมบัติที่เหมาะสมในการพัฒนาเป็นวัสดุปิดทับเนื้อเยื่อในฟันมนุษย์ เนื่องจากสามารถฆ่าเชื้อเสตร็ปโตคอคคัสมิวแทนส์ และแลคโตบาซิลลัสคาเซไอซึ่งเป็นเชื้อที่พบบ่อยในเนื้อเยื่อในของฟันมนุษย์ที่ติดเชื้อ และไม่เป็นพิษต่อเซลล์เนื้อเยื่อในฟันมนุษย์

คำสำคัญ : สารสกัดพรอพอลิสไทย, องค์ประกอบทางเคมี, ฤทธิ์ต้านจุลชีพ, ความเป็นพิษต่อเซลล์

Phenolic and flavonoids contents and antibacterial activity of ethanolic extract of propolis.

Pattama Chailertvanitkul[1], Thitima Namsirikul[2], Teerasak Damrongrungruang[3] and Jomjai Peerapattana[4]*

[1]Associate Professor, Department of Restorative Dentistry, Faculty of Dentistry, Khon Kaen University, Khon Kaen 40002, Thailand

[2] Dentist, Kamphaeng Saen Hospital, Nakhon Pathom, 73140, Thailand

[3] Associate Professor, Department of Oral Diagnosis, Faculty of Dentistry, Khon Kaen University, Khon Kaen 40002, Thailand

[4] Associate Professor, Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

*** Corresponding author :** Tel.: +66 86 862 9401; fax: +66 43 362 092. E-mail address: jomsuj@kku.ac.th

Abstract

Phenolic and flavonoids contents and antibacterial activity of ethanolic extract of propolis.

Pattama Chailertvanitkul[1], Thitima Namsirikul[2], Teerasak Damrongrungruang[3] and Jomjai Peerapattana[4]*

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The aim of this work was to study properties of Thai propolis extract (TPE) for the development of dental pulp capping material. Raw propolis materials were collected from Nong Khai province and extracted using 95% ethanol. Total flavonoid contents were determined by Aluminum chloride colorimetric method and 2,4-Dinitrophenylhydrazine colorimeteric method. Total phenolic compounds were determined using Folin-Ciocalteau colorimetric method. The results showed that TPE had flavonoids 10% (w/w of TPE) and phenolic 2.95% (w/w of TPE). TPE had an inhibition effect against both *Streptococcus mutans* and *Lactobacillus casei*. The minimum inhibitory concentration of TPE was 22.5 mg/ml and 11.25 mg/ml for *Streptococcus mutans* and *Lactobacillus casei*, respectively. The minimum bactericidal concentration of TPE was 45 mg/ml for both bacteria. TPE concentration 90 mg/ml (two-fold of minimum bactericidal concentration which planned for pharmaceutical preparation) had no cytotoxicity on human dental pulp cells at 6, 12, 24 and 72 hours**.** The results of this study suggest that TPE is suitable for development of dental pulp capping material because it had bactericidal effect against *Streptococcus mutans* and *Lactobacillus casei* which were often found bacteria in infected human dental pulp and it had no cytotoxicity on human dental pulp cells.

Keywords: Thai propolis extract, chemical composition, antimicrobial activity and cytotoxic effect

Introduction

Dental caries is one of the most prevalent diseases affecting the calcified tissues of the teeth. Although many acid producing microbes have been isolated from oral flora, *Streptococci* and *Lactobacilli* have been considered as significant ones in contributing to the initiation and progression of deep dental caries respectively and diffusing through tubular dentine to pulp chamber (Martin *et al.*, 2002; Munson *et al.*, 2004; Chhour *et al.,* 2005; Featherstone 2008; Ophori *et al.,* 2010; Neelakantan *et al.,* 2012). Removal of softened dentin would further involve pulp exposure, and hence it is imperative to preserve the vitality of the pulp by meticulous conservative approach by using effective medicaments rather than performing root canal treatment. Vital pulp therapy is the choice of treatment for

maintain pulp vitality and function of dentine-pulp complex (Tziafas *et al.,* 2000). It consists of indirect pulp capping, direct pulp capping, partial pulpotomy and full pulpotomy. Success of vital pulp therapy depends on operative procedure to remove infection, treatment modality, materials and bacterial-tight seal restoration which promote pulp tissue healing and repairing (Kakehashi *et al.*, 1965; Mejare and Cvek 1993).

Calcium hydroxide is a gold standard of pulp capping material for vital pulp therapy. There are many advantages such as bactericidal, activated formation of reparative dentine, easy to use, long-term success rate. However, this material has some drawbacks. Firstly, it lacks dentin adhesive properties due to its softness and solubility (Pereira *et al.*, 1990). Secondly, the dentin bridge induced by this material is porous hence there is potential for bacterial penetration (Kitasako *et al.,* 2008). Thirdly, calcium hydroxide has limited antibacterial properties toward *E. faecalis, Candida* and *Actinomyces* species which are associated with persistent disease after root canal treatment (Cox *et al.*, 1996). Lastly, calcium hydroxide is toxic to pulp cells (Al-Shaher *et al.,* 2004). Due to these disadvantages, a superior material has been sought after. Mineral trioxide aggregate (MTA) is recently candidate for pulp capping material. It is biocompatible, bactericidal, well sealing ability and hard tissue inductive. However, it was not widely used because of expensive cost, long time setting and few long term studies.

At present, the study of the use of extract from natural origin is increasing. Propolis, a natural extract of beehives, consists of various chemical compounds depending on bee strain, local flora, geographic and season. Main chemical compounds are flavonoids, phenolic and aromatic compounds (Kumazawa *et al.*, 2004; Parolia *et al.,* 2010). Propolis has antimicrobial activities (Ikeno *et al.,* 1991; Uzel *et al.,* 2005; Mohammadzadeh *et al.,* 2007; Liberio *et al.*, 2009), anti-inflammatory (McLennan *et al.*, 2008) and low toxicity (Burdock 1998; Al-Shaher *et al.,* 2004). There are many studies of propolis extract for dental use including direct pulp capping material (Parolia *et al.,* 2010; Ahangari *et al.,* 2012). However, there was not a report of Thai propolis extract (TPE), therefore, the aim of this study was to investigate the chemical compositions, antibacterial properties and cytotoxicity effect of TPE.

Materials and methods

1. Extraction of Thai propolis

Raw Thai propolis materials were collected from *Apis mellifera* bee-hives (Figure 1), fed with corn and banana, from Nong-Khai province, Thailand. It was chopped into small pieces and extracted using 95% ethanol (1 g: 5 ml) in orbital shaker incubator (MRC, Holon, Israel) at 25ºC, 200 rpm for 5 days. The mixture was filtered through Whatman™ no.1 filter paper (Whatman Inc., Piscataway, USA), stored at -20ºC for 48 hours and then filtered again. The filtrate was evaporated using rotary evaporator (Eyela, Tokyo Rikakikai, Tokyo, Japan) at 40ºC and stored at 4ºC, protected from light. The concentrated filtrate was dried using freeze dryer (Alpha 2-4 LD plus, Christ, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and the percentage of yield was calculated. The product was named Thai propolis extract (TPE).

Figure 1 Raw Thai propolis materials.

2. Determination of flavonoid and phenolic contents

2.1 Total flavonoid contents

Flavonoids consist of 4 major chemical groups i.e. flavones and flavonols reacted well with aluminum chloride colorimetric method, and flavanones and flavanonols reacted better with 2,4-Dinitrophenylhydrazine. Aluminum chloride colorimetric method was modified from the procedure described by Chang *et al.*, (2012). TPE was diluted to 20 mg/ml in absolute ethanol. Aliquots of 0.5 ml of quercetin (Calbiochem, EMD chemicals, Germany) was mixed with absolute ethanol 4.3 ml, 10% aluminum chloride 0.1 ml (QRëC™, QREC (Asia) SDN BHD, Malaysia) and 1 M of potassium acetate 0.1 ml (QRëC™, Brightchem SDN BHD, Malaysia). Mixed solution was stored at 25ºC for 30 minutes. The light absorbance of the solution was measured at wavelength 415 nm using multifunction microplate reader (Varioskan Flash, Thermo Fisher Scientific, USA). The light absorbance of quercetin (Calbiochem) 60, 90, 120, 150 and 180 µg/ml in absolute ethanol was also measured and the standard calibration curve was plotted.

Flavanones and flavanonols were determined by 2,4-Dinitrophenylhydrazine colorimeteric method modified from the procedure described (Cvek *et al.*, 2007). TPE was diluted to 20 mg/ml in absolute ethanol. Aliquots of 1 ml of (±)-naringenin (Sigma-Aldrich, USA) was mixed with 1% 2,4-dinitrophenylhydrazine 2 ml and methanol 2 ml, incubated at 50ºC for 50 minutes. After cooling, 10% potassium hydroxide (QRëC™, Brightchem SDN BHD, Malaysia) in 70% methanol 5 ml was mixed at 25ºC for 2 minutes. The resulting solution 1 ml was mixed with methanol 5 ml and centrifuged at 1,610 g for 10 minutes using centrifuge machine (Sorvall® RC 6 plus, Thermo Scientific™, Thermo Fisher Scientific, USA). The light absorbance of supernatant was measured at wavelength 495 nm using multifunction microplate reader. The light absorbance of (±)-naringenin (Sigma-Aldrich) 1,200, 1,400, 1,600, 1,800 and 2,000 µg/ml in absolute ethanol was also measured and the standard calibration curve was plotted.

2.2 Total phenolic compounds

Total phenolic compounds were determined by Folin-Ciocalteau colorimetric method modified from the procedure described elsewhere (Vitchayakitti *et al.,* 2009). TPE was diluted to 20 mg/ml in absolute ethanol. Aliquots of 0.1 ml of gallic acid (Sigma-Aldrich, USA) was mixed with Folin-Ciocalteau reagent (Carlo Erba Reagents, Rodano, Milan, Italy) 0.5 ml and distilled water 7 ml. Mixed solution was kept at 25ºC for 8 minutes then 20% Sodium carbonate (Univar, Ajax Finechem Pty Ltd, Australia) was added and incubated for 2 hours in dark room. The light absorbance of supernatant was measured at wavelength 765 nm using multifunction microplate reader. The light absorbance of gallic acid (Sigma-Aldrich) solutions 400, 450, 500, 550 and 600 µg/ml in absolute ethanol were measured and the standard calibration curve was plotted.

3. Antimicrobial activity

Bacterial strains used in this study were *Streptococcus mutans* ATCC 25175T (Department of Medical Sciences Culture Collection, Bangkok, Thailand) and *Lactobacillus casei* BCC 36987 (Thailand Institute of Scientific and Technological Research Culture Collection, Bangkok, Thailand). Preparation of inoculums were modified from method for dilution antimicrobial susceptibility test for bacteria that grow aerobically (Clinical and Laboratory Standards Institute 2006). *S. mutans* and *L. casei* were transferred from the stock culture to Mitis Salivarius agar (BD, Becton, Dickinson and company, France) and de Man, Rogasa and Sharpe agar (Himedia®, HiMedia Laboratories, India) respectively with Cross-streak technique and incubated at 37ºC for 24 hours. Single colony of *S. mutans* was transferred to the Todd Hewitt broth (BD, Becton, Dickinson and company, France) and incubated at 37ºC for 24 hours. Single colony of *L. casei* was transferred to de Man, Rogasa and Sharpe broth (Himedia®, HiMedia Laboratories, India) and incubated at 37ºC for 48 hours. The suspension was adjusted to the McFarland 0.5 turbidity standard (1.5 x 10^8 CFU/ml).

Antimicrobial susceptibility test of TPE was investigated by agar disc diffusion method modified from Kashi *et al.*, (2011). Petridishes (100 mm diameter) were filled with Mitis Salivarius agar and de Man, Rogasa and Sharpe agar for *S. mutans* and *L. casei*, respectively. Sterile cotton swabs were dipped into the inoculum of each suspensions and three-dimension swabbed to agar surfaces. Aliquots of 20 µl of TPE, absolute ethanol and 2% chlorhexidine digluconate were dropped on paper discs (6 mm diameter and 1 mm thick) then placed on the agars plates and incubated at 37ºC for 24 and 48 hours for *S. mutans* and *L. casei*, respectively. Absolute ethanol was used as a negative control and 2% chlorhexidine digluconate was used as a positive control. Inhibition zones (the diameter of outside margin of microbial growth minus the diameter of the paper discs) were measured 3 times. The tests were performed three replicates for each bacteria.

The minimum inhibitory concentration (MIC) of TPE was investigated by two-fold serial dilution method modified from Kashi *et al.,* (2011). Aliquots of 100 µl TPE was diluted with Todd Hewitt broth for *S. mutans* and de Man, Rogasa and Sharpe broth for *L. casei* and added with 100 µl of each bacterial suspensions McFarland 0.5 turbidity standard. The sterile broth 200 µl was used as a negative control. The sterile broth 100 µl with 100 µl of each bacterial suspensions McFarland 0.5 turbidity standard was used as a positive control. The plates were incubated at 37ºC, 5% CO_2 for 24 and 48 hours for *S. mutans* and *L. casei*, respectively. The light absorbance was measured at wavelength 600 nm using multifunction microplate reader. The tests were performed triplicate for each bacteria.

The minimum bactericidal concentration (MBC) of TPE was investigated by method modified from Kashi *et al.,* (2011). Aliquots of 5 µl of various concentrations of TPE which no visible growth of bacteria from previous tests were inoculated to the Mitis Salivarius agar for *S. mutans* and de Man, Rogasa and Sharpe agar for *L. casei*. The plates were incubated at 37ºC, 5% CO_2 for 24 and 48 hours for *S. mutans* and *L. casei*, respectively. The tests were performed triplicate for each bacteria.

4. Cytotoxicity of TPE

This study was approved by the office of the Khon Kaen University ethics committee to human research. Human dental pulp cells were obtained from extracted sound human premolar tooth. The tooth was immediately immersed in Dulbecco's Modified Eagle Medium (BioWhittaker®, Lonza Walkersville, Walkersville, USA) with 10% fetal bovine serum (BioWhittaker®, Lonza Walkersville, Walkersville, USA), penicillin G (BioWhittaker®, Lonza Walkersville, Walkersville, USA) 100 unit/ml, streptomycin (BioWhittaker®, Lonza Walkersville, Walkersville, USA) 100 µl/ml and amphotericin B (BioWhittaker®, Lonza Walkersville, Walkersville, USA) 25 µl/ml. The tooth was splitted longitudinally and pulpal tissue was cut into small pieces and cultured in tissue culture dish with the medium at 37ºC, 5% CO_2 and 95% humidity. Dental pulp cells were subcultured and cells between third to eighth passage were used (Chaipanha *et al.*, 2013).

The dental pulp cells (2×10^5 cells/ml) in 50 µl Dulbecco's Modified Eagle Medium (BioWhittaker®) with 10% fetal bovine serum (BioWhittaker®) were incubated at 37ºC, 5% CO_2 and 95% humidity for 24 hours. The medium was changed to serum-free medium twice every three hours and incubated for 24 hours. Dental pulp cells were cultured in TPE solution at concentrations of MBC and 2-fold of MBC against *S. mutans* or *L. casei* respectively at 6, 12, 24 and 72 hours. Dental pulp cells cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine were used as a positive control group and pulp cells cultured in 10% hydrogen peroxide were used as a negative control group. At each test period, dental pulp cells in each solution were rinsed with phosphate buffer saline (PBS) and added with Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. Cytotoxicity effects were evaluated using manual counting method with inverted microscope (Axiovert 25, Zeiss Frankfurt, Germany) at 100X by capturing the same areas in each well with NIS Elements BR 3.00 imaging software (Nikon Instruments). Dental pulp cells per area 1 cm^2 were counted twice.

Results

Extraction of Thai propolis

Raw Thai propolis (Figure 1) was sticky mass with dark brown color. The yield of this extract was 3.67% (w/w). TPE was also dark brown color with unique smell.

Determination of flavonoid and phenolic contents

TPE contained flavonoids 10% (w/w) which composed of flavones and flavonols 0.73% (w/w quercetin equivalent) and flavanones and flavanonols 9.27% (w/w naringenin equivalent). The extract had phenolic compounds 2.95% (w/w gallic acid equivalent) (Table 1).

Table 1 The flavonoids and phenolic compounds content of TPE determined by colorimetric methods.

Flavonoids and phenolic compounds	Percentage (w/w)
Flavones and flavonols (quercetin equivalent)	0.73
Flavanones and flavanonols (naringenin equivalent)	9.27
Phenolic compounds (gallic acid equivalent)	2.95

Antimicrobial activity

The extract had antimicrobial activities. Average inhibition zone of TPE against *Streptococcus mutans* was 8.31±0.06 mm and against *Lactobacillus casei* was 3.55±0.09 mm. Minimum inhibitory concentration (MIC) against *Streptococcus mutans* was 22.5 mg/ml, against *Lactobacillus casei* was 11.25 mg/ml. Minimum bactericidal concentration (MBC) against both bacteria was 45 mg/ml (Table 2).

Table 2. Inhibition zone, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of TPE, positive and negative controls.

	Inhibition zone (mean ± S.D.) (mm)			MIC (mg/ml)	MBC (mg/ml)
	TPE	2% Chlorhexidine digluconate	Absolute ethanol		
S. mutans	8.31 ± 0.06	6.62 ± 0.08	0	22.5	45
L. casei	3.55 ± 0.09	16.91 ± 0.15	0	11.25	45

Cytotoxicity of TPE

Cytotoxicity of TPE was tested at MBC (45 mg/ml) and 2-fold of MBC (90 mg/ml) since this study plan to prepare Thai propolis preparation at concentration 2-fold of MBC. TPE both concentrations showed no cytotoxicity and stimulated human dental pulp cells in every test durations. At 6, 12, 24 and 72 hours, TPE concentration 45 mg/ml showed no cytotoxicity and could stimulate human dental pulp cells proliferation between 99.57-106.93 % whilst at concentration 90 mg/ml it could stimulate human dental pulp cells proliferation between 101.28-112.22 % (Figure 2).

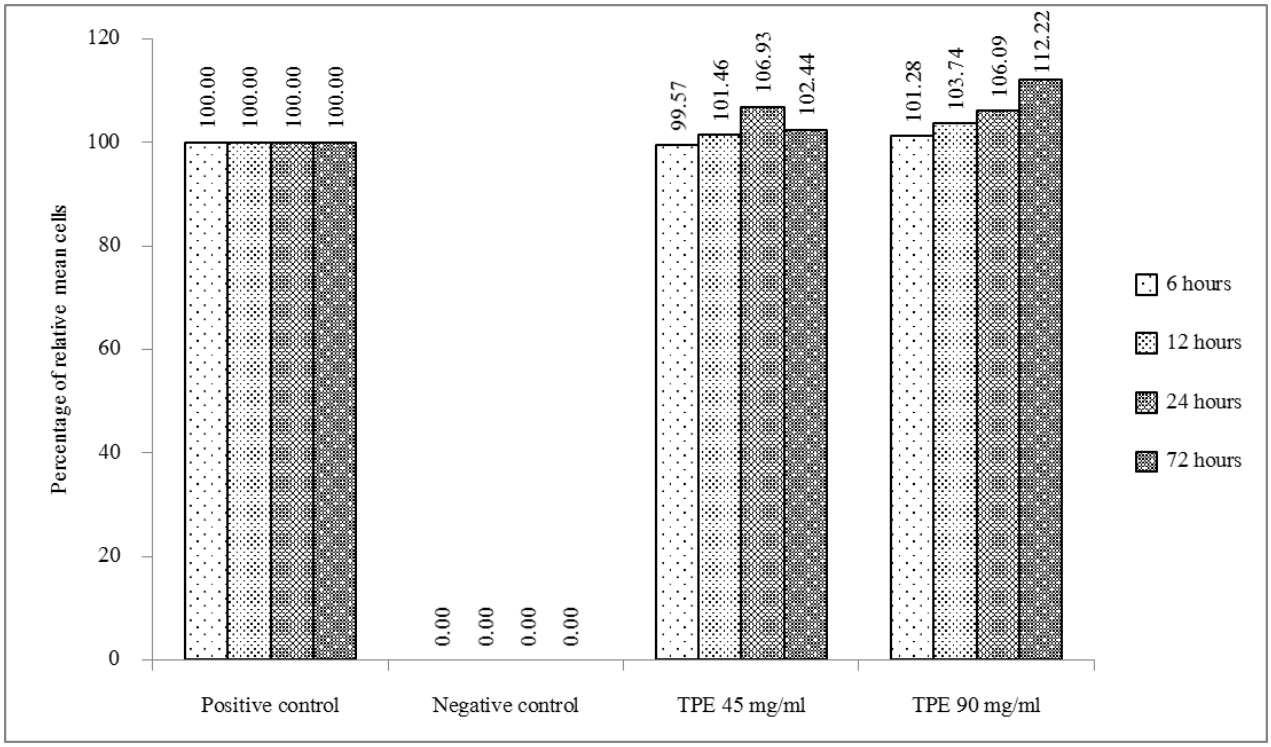

Figure 2. Comparison of percentage of average human dental pulp cells number between 45 mg/ml, 90 mg/ml of TPE with positive and negative control after 6, 12, 24 and 72 hours treatment.

Discussion and Conclusions

TPE percentage of yield in this study was less than Anatolian propolis, studied by Uzel and co-worker (2005), which were in the range of 20.51 – 44.80 % (w/v). Flavonoids and phenolic compounds of propolis extract had been determined by many different methods including high performance liquid chromatography (Kumazawa *et al.*, 2004; Daugsch *et al.*, 2008; Siripatrawan *et al.,* 2013), gas chromatography–mass spectrometry (Uzel *et al.*, 2005; Mohammadzadeh *et al*., 2007) and colorimetric method (Chang *et al.*, 2002; Cvek *et al.*, 2007; Siripatrawan *et al.*, 2013). Colorimetric method is considered convenient, not required advanced instruments and low cost, therefore, they were used in this study to detect both flavonoids and phenolic compounds. Total flavonoids contents of TPE were 10% (w/w) which were higher than a study by Kumazawa *et al.,* (2004). Total phenolic compounds were 2.95% (w/w) which were similar to a study by Kumazawa *et al*., (2004). Ethanolic extraction at room temperature give higher flavonoids and phenolic compounds than that water extraction (Mello and Hubinger 2012; Kaewmuangmoon *et al*., 2012; Siripatrawan *et al*., 2013). This study used ethanolic extraction at room temperature for the best results.

TPE has antimicrobial activities. Siripatrawan *et al*., (2013) found that TPE kill Gram positive bacteria better than Gram negative bacteria. Our research studied 2 Gram positive bacteria mostly found in carious lesion. The MIC and MBC of TPE was higher than Iranian propolis extracts which were only 250 µg/ml (Kashi *et al*., 2011). The inhibition zone of TPE was wider than chlorhexidine digluconate for *S. mutans* but less than chlorhexidine digluconate for *L. casei*.

It is noticeable that there were different in percentage of yield, flavonoids and phenolic compounds, MIC and MBC of TPE compared with other propolis. These could be due to the different source of starting propolis materials, seasonal variation of harvest and extraction method (Kaewmuangmoon *et al*., 2012, Nunes *et al*., 2013; Siripatrawan *et al.,* 2013).

Although MTT cytotoxicity assays was a common cell counting technique. Our previous study (Chaipanha *et al.,* 2013) found that the yellowish color of TPE was similar to MTT reagents and had an impact on the determination of optical density by spectrophotometer. In this study, alamar blue cytotoxicity assay was used because of the blue color reagent gave different color of solution, no cytotoxicity and more sensitive than MTT cytotoxicity assays. Viable cells do not take trypan blue dye appear unstained while nonviable cells have no intact cell membrane appeared blue stained (Bio-Rad Laboratories 2011). This study, viable cells was counted manually using phase contrast microscope because it was simple, no need for special or complicated equipments. This study counted cells twice, statistical analysis showed that the count was consistency (Kendall rank-order correlation coefficient; τ= 0.855, $P < 0.001$), and triple checked with an expert.

TPE contained 10 % flavonoids and 2.95 % phenolic compounds and had antimicrobial activities against *S. mutans* and *L. casei*. TPE concentration 90 mg/ml had no cytotoxic effect and can stimulate human dental pulp cells *in vitro*. The results of this study suggest that TPE is suitable for development of dental pulp capping material because it had bactericidal effect against *Streptococcus mutans* and *Lactobacillus casei* which were bacteria in human dental palp and it had no cytotoxicity on human dental pulp cells.

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