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RESEARCH ON THE COMPOSITION AND CYTOTOXIC ACTIVITY OF PINUS BRUTIA GUM

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ABSTRACT

Pinus Brutia Ten., traditionaly used as sugar regulator, antioxidant and antitumoral is consumed by chewing in Turkey. The aim of the study is to investigate the components and the effect of the gum. The base of the study depends on the way of comsumption of the resin. For in-vitro analysis, artificial salvia is used. The samples is stayed in artificial salvia at 37° C in ultrasonic bath for different time period and then determined by U-HPLC, PDA detector. Three samples were prepared from each sample and three injections were made. Caffeic acid was investigate in the samples. The optimum waiting period is set at 5 hours, which is considered to be a possible period since the gum adheres to the tooth and is a hardly soluble substance. Considering the anticarcinogenic effect of caffeic acid, cytotoxic test was carried out on specimens which were kept in saliva for 24 hours acid. After incubation of the saliva extract that including 10, 20, 50 µM caffeic acid in liver cancerous cells 1, 3, 6 hours, cell viability was observed.

1. INTRODUCTION

Pinus brutia Ten. resin is known as pine gum because it can be chewed like gum among the people in many regions of Turkey. Among the people the gum is used as antiseptic, sugar regulator, and also in Ottoman time, the compound was used in the composition of the mixtures that used for tumor healing (Arıtuluk *et al*., 2012, Saçlı *et al.,* 2001 and Atıcı 2007). There are not many published scientific studies on resins. Resin-based studies are more like volatile oil analyzes (Ulukanlı *et al*., 2014, Avnı *et al*., 2016).

A small number of active substance analyses are done on the different pine tree shells. Various studies have shown that the pine tree shells have beneficial effects on inflammation, C-deficient scurvy disease and flavonoids in the immune system diseases, glucose metabolism (Ince *et al.,* 2009, Maimoonae *et al,*2011, Kim *et al*., 2004 and Kim *et al.,* 2005). *Pinus* species have economic importance in pharmaceutical and cosmetic sectors. For instance; turpentine has been known to have a long record of remedial utilization primarily as topical counter irritants for the treatment of rheumatic disorders and muscle pain. Pine bark extract is also used in anti-aging cosmetics (Yonei *et al.,* 2004)

In Chinese medicine, pine resin is used for the treatment of skin diseases and burn scald wounds (Yang *et al.,* 2010). There are few reports are available on chemical components of P *Brutia resin* in Mediterinian countries (Satil *et al*., 2011, Iconomou *et al.,* 1964, Schiller *et al.,* 1987). The poisonous effect of the material on organism is determined by cytotoxic assay. Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss *et al*., 2013). There is no cytotoxic study on *Pinus* resin. The resin is used as a gum and while chewing it sticks the teeth. The main aim of the study to analyse the content and the amount of the gum is solved in salvia in different time period.

The other aim of the study is to analyse the effect of pinus gum which is solved in salvia on cell vitality.

2. MATERIAL AND METHODS

2.1. Instumentation

U-HPLC equipped with Thermo Scientific, Accela Model 1250 Pump, Autosampler ve PDA dedector is used for the ingredient analysis.

2.2. Material and Reagents

The resin was bought from markets and the identification was done Ass. Prof. Zeki Haznedaroglu, Katip Çelebi University, Faculty of Pharmacy, Department of Pharmaceutical Botany. The resin was milled and put into plastic tubes to react 5 mL artifical salvia (0.2 g.K_2HPO_4, 0.330g. KSCN,0.260 g. Na_2HPO_4, 1.500 g. $NaHCO_3$,0.700g. NaCl, 1.200g. HCl,1.300g.urea in L, pH=4) (Can *et al*., 2006). Merck branded chemicals was used for the preparation of artificial salvia. The tubes were shaken and left in the ultrasonic bath for 1, 2, 5,10 minutes and 5,10,18, 24 hours at 37^{O}C. Three of each sample were prepared. Full time samples were filtered through the filter paper.

Merck branded chemicals, methanol (HPLC gradient, J.T. Baker 8402), o-phosphoric acid (Redel-de Haen 30417) and bi-distilled water were used for mobile phase. Distilled water was used after filtered with 0.22 µm pore diameter Millipore Express©- PVDF vacuum filter unit. 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium MTT bromide was obtained from Sigma. Standards used in HPLC analysis were obtained from Sigma. 5891 Schwarzband 125 mm filter paper is used also.

2.3. Standard Preparation

Stock solution of dihydroxybenzoic acid, caffeic acid, t-ferrulic acid and m-cumaric acid were preperad in methanol (1mgmL^{-1}). 6, 8, 10 µgmL^{-1}diluted mixtures of the solutions were used for working standards. The mixtures were injected at three times (n: 3). Merck branded standard were used for the analysis.

2.4. Cell Viability: MMT Cytotoxicity Assays

300 mg milled resin gum waited in artificial salvia for 24 hours, at 37^{O}C in ultrasonic bath. This extract that contains 10, 20, 50 µM caffeic acid were incubated for 1, 3, 6 hours in liver cancer cells and cell viability was detected. (Kim *et al.,* 2015). For each hour 3 measurement were done.

Percent cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mossman *et al.,* 1983). MTT (5 mg / ml) was prepared in 5 ml PBS in falcon. It was stored in the dark and at -20 ° C. 96 well culture plate (flat bottom), 2x10 ^{3} cells / well, 150 µl / well were added, to stick to the cells for 24 hours were kept. Used medium: DMEM / F12 + 10% FBS + 100 U / ml penicillin + 100 U / ml streptomycin. A mixture of 10.63 µL (10 µM) or 21.27 µL (20 µM) or 53.15 µL (50 µM) was added to the wells without media removal and incubated for 1, 3 and 6 hours at 37°C in a CO_2 humidified incubator. 20 µl MTT (5 mg / ml in PBS) was added to each incubator and incubated at 37° C in a CO_2 incubator for 3 hours until the formation of purple crystals. Then, 100 µl dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken at room temperature for 1 h and then the absorbance was measured at 570 nm with a reference setting of 650 nm using a microplate reader (Mossman, *et al.,* 1983, Bruggisser, *et al*., 2002).

Calculation: Aresult = A570-A650. The Ablank is removed from the resulting value. % Cell viability is calculated**.**

3. RESULTS AND DISCUSSION

The first step of an analysis is to determine the extraction method. There is no study for Pinus resin extraction. In Turkey, traditional use of *Pinus brutia* Ten resin is chewing, therefore the study depends on the extract of salvia and finding the active ingredients of the gum that pass through the salvia.

There is no information about the contents of the prepared samples, two mixture standards were prepared and measured by U-HPLC. Preliminary studies were carried out by comparing the chromotograms obtained by injection of 24 hour samples with mixture standard chromotograms, to determine possible agents. Based on the recovery time, it was decided to evaluate samples against 6-10 µg / mL 3,4-dihydroxybenzoic acid, caffeic acid, t-ferulic acid, m-coumaric acid standards. The LOD and LOQ Limits of the samples are given in Table 1.

Table 1. Limit of dedection and limit of quantitation of the standards.

Standards	Retantion time (RT), min	Wave lenght, nm	LOD, µg/mL	LOQ, µg/mL
3,4_dihydroxysibenzoicacid	5.73	295	~ 0.5795	~ 1.9317
Caffeic acid	8.02	324	~ 0.3279	~ 1.0931
t-ferrulic acid	10.04	322	~ 0.5076	~ 1.6920
m-cumaric acid	10.50	278	~ 0.1533	~ 0.5112

In chromatogram analysis, the recovery and wave length of the chromatogram of standards and 24 hour salvia extract were compared and caffeic acid was found in gum extract. There were different peaks in the sample chromatogram which can not be determined. Chromatograms of 10 µg / mL standard mixture and 24 hour extracted gum samples were given in Figure 1. In the analysis done by U-HPLC, the presence of caffeic acid was determined. Some studies have shown that caffeic acid is found in pine bark (Yesil Çeliktas *et al.,* 2010). However, if there is no information about the content of the resin, it is necessary to make the comparison with the pine bark.

Possible pharmacological effects of caffeic acid have been shown in several studies. Rahenda Prasad et al. (2011) reported caffeic acid is reduced cell degeneration in cancerous tissue, antioxidant and immunomodulator and it has anti-inflammatory properties (Olthof *et al*., 2001, Oroion *et al*., 2015, İlhami, 2006 and Oktan *et al*., 2010). In addition, the effects of caffeic acid in diabetic mice were investigated (Durmus *et al*., 2008). It is noteworthy that traditional the reasons for the use of resin gum overlap with these effects

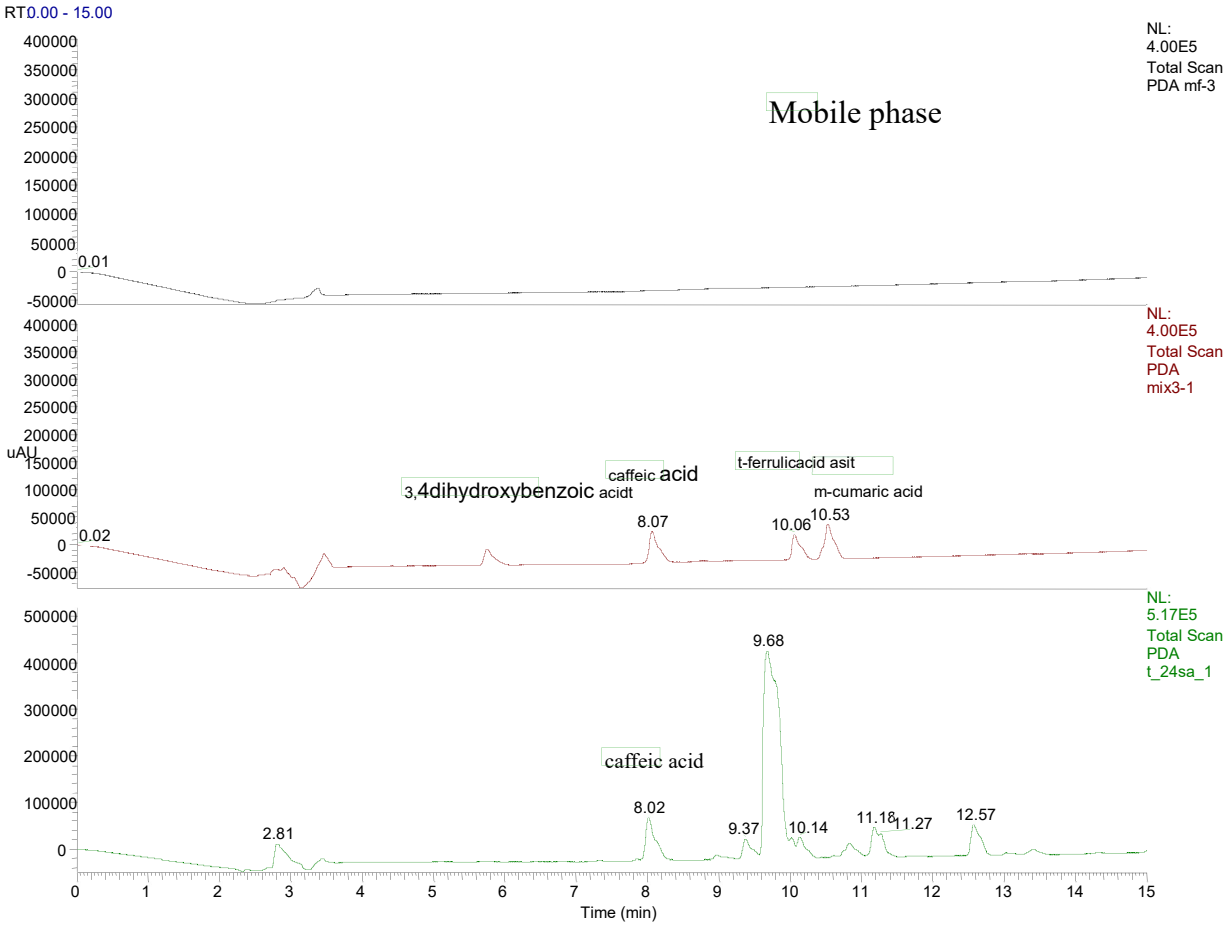


Figure 1: Chromatogram of mobile phase, standards mixture and artificial salvia that gum stayed for 24 hours.

To determine the caffeic acid quantity that passed through the salvia from resin, the same quantity of the resin were stayed 1, 2, 5, 30, 60 minutes and 5, 10, 18, 24 hours at 37 °C in ultrasonic bath in artificial salvia and were analysed by UPLC in triplicate. The calibration equation is found as y= 31954,9 + 216040 and r^2= 0.999. The results is shown in Table 2. The amount of caffeic acid passing through the salvia varies with time is shown in Figure 2.

Table 2: Caffeic acid concentration of the resin extracts

Time	Concentration µg/mg
1 min	0.0939 ± 0.0058
2 min	0.0399 ± 0.0001
5 min	0.0176 ± 0.0007
10 min	0.1177 ± 0.0218
30 min	0.0972 ± 00179
60 min	0.1093 ± 0,0001
5 hour	0.6810 ± 0.1272
10 hour	0.4038 ± 0.1075
18 hour	0.3757 ± 0.0341
24 hour	0.7064 ± 0.1964

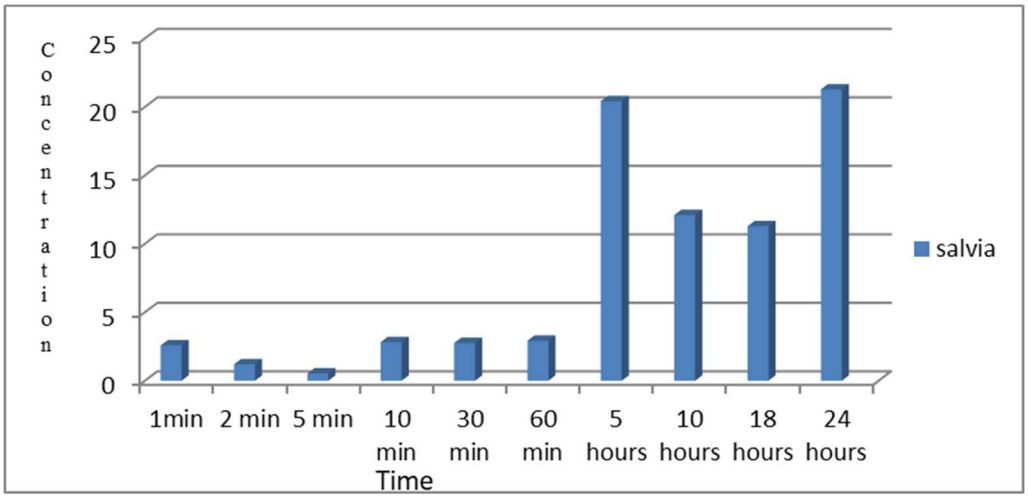


Figure 2: Changes in the amount of caffeic acid concentration (µgmg^{-1}) passing from resin to salvia

The amount of caffeic acid passing through the salvia varies with time. When Figure 2 is examined, it is found that the passage of the active substance from the sample is getting higher after 5 hours. The resin is insoluble in saliva and forms a solid at the bottom. The 1 minute sample was higher than the 2 and 5 minute samples, it was thought that at the beginning the gum was in powder form and in minutes getting harderer and active material release were

getting down. In hours the concentration is getting higher. This can be explained by the breakage of the outer shell of the hard resin. In MMT test results, different concentration of the resins were increased the cell quantity and stopped the cell death in cell culture in 1, 3, and 6st hours. The results are given in Figure 3. The microscopic photographs of the cristals in cells at 3 and 6 hours are given in Picture 1 that is shown the cell viability.

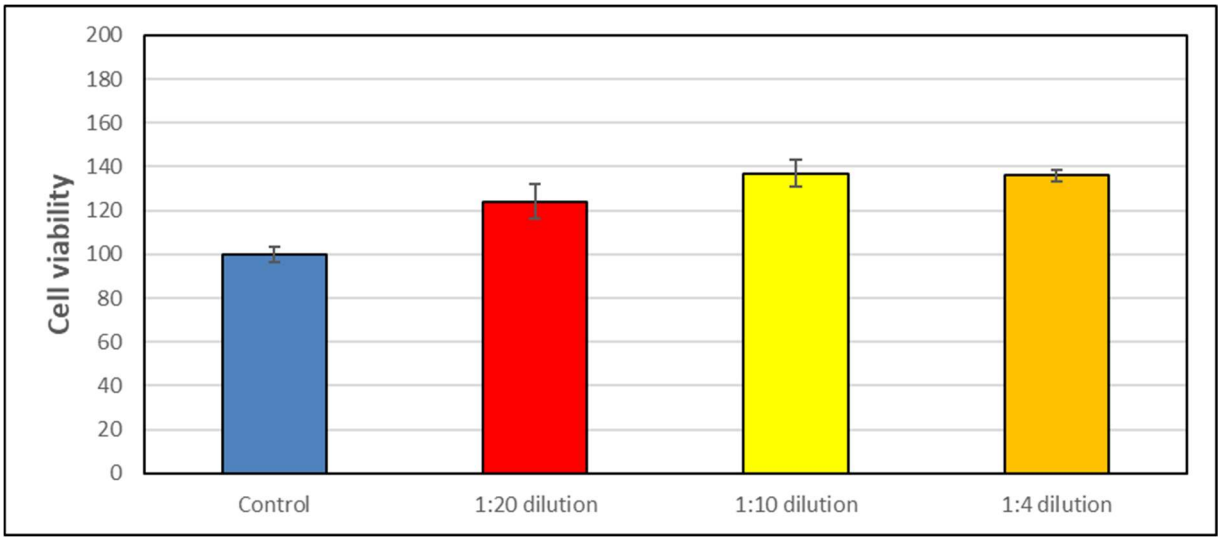
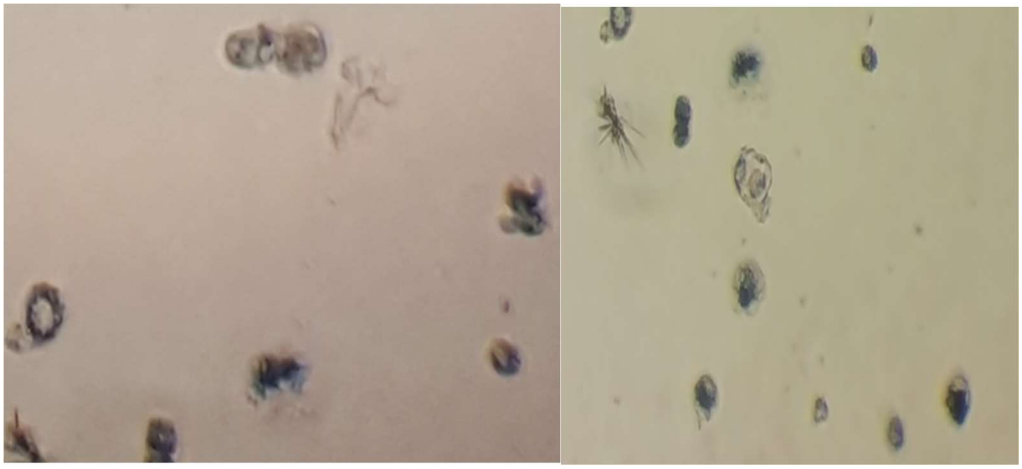


Figure 3: Cell viability results of control group and different concentration of the gum.



Picture 1. 3 hours photo at left and 6 hours photo is at right (The purple inside of the cells are the proof of the cell viability)

4. CONCLUSIONS

It is determined that the pinus resin that used as a chewing gum in public contains various active ingredients. Caffeic acid and the amount of caffeic acid in artificial salvia that passed from the resin is observed. The result of cell culture studies show that chewing gum is hepatoprotective. We can say that these kinds of products are both a protective raw material and a natural product because they are both a pharmaceutical raw material and a preferred product in preventive medicine.

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