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Spectroscopy Structure Analysis of Ellagic Acid and Calcium Phosphate

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Abstract

Ellagic acid is a polyphenol found in pomegranates. It contains a stimulant of osteoblast genesis and anti-inflammatory properties, and it is osteo inductive. Bovine bone xenograft is also osteoconductive and becomes one of the properties of the bone graft matrix that supports the attachment of bone-forming cells. Fourier transform infrared (FTIR) is an instrument that uses spectroscopic principles. Infrared spectroscopy is useful for identifying organic compounds because of its very complex spectrum consisting of many peaks. This study determined the chemical bonds of ellagic acid, calcium phosphate, and their combination to the spectrum of functional groups produced. FTIR characterization was carried out with ellagic acid powder, bovine bone xenograft powder, and a combination of samples with a ratio of 50:50. The FTIR results from the combination of ellagic acid and calcium phosphate showed a shift in wavenumber. This research shows the combination of calcium phosphate and ellagic acid contain different functional groups. When combined, the compounds are still strong in ellagic acid.

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Introduction

Pomegranate or *Punica Granatum Linn.* (PGL) is among the most widely studied medicinal plants for health purposes. Pomegranates' antioxidant and anti-inflammatory activity may occur due to their high polyphenol content.[1] The polyphenols contained in pomegranate include ellagic acid (EA), gallotannins, and anthocyanins, among others.[2] Ellagic acid is considered as a stimulant of osteoblast genesis, and it is osteo inductive. Besides, it has anti-inflammatory properties.[3]

To produce materials that can support bone tissue regeneration, a synergistic combination of two ingredients i.e., ellagic acid and bovine bone xenograft necessarily provides optimal function in the form of a synergistic effect on the process of new bone growth along with bone formation. Bovine bone xenograft is osteoconductive as the nature of the bone graft matrix supports the attachment of bone-forming cells,[4] while ellagic acid is osteo inductive because it stimulates the differentiation of osteoprogenitor cells into osteoblast cells.[3]

One of the many integral analytical methods used by researchers is fourier transform infrared (FTIR). This technique distinguishes liquids, solutions, pastes, powders, films, fibers, and gases. It can also be used to analyze materials on the surfaces of substrates[5]. FTIR is fairly popular when compared against other characterization analysis techniques. This method is quite quick, highly accurate, and relatively sensitive.[6] Samples come into contact with infrared (IR) radiation for the FTIR procedure. The atomic vibrations of the samples' molecules are impacted by the IR radiation, producing the specific absorption and/or transmission of energy. With the IR radiations, the FTIR is functional for identifying specific molecular vibrations in the samples.[7] This current report discussed and explained the chemical bonding of calcium phosphate, ellagic acid, and their combination to yielding functional group spectrums.

Materials and methods

This study was conducted at the Metallurgical Laboratory of the Sepuluh

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November Institute of Technology Surabaya in August to October 2019. The main ingredient used was ellagic acid, produced by Xi'an Biof Bio-Technology Co., Ltd. (Room 1-1111, High-tech Venture Park, No. 69 Jinye Road, Gaoxin District of Xi'an, People Republic of China). Bovine bone xenograft is a xenograft material that has been irradiated with rays and has its immunogenic and soft tissue properties removed. The xenograft was made at the Tissue Bank of District General Hospital of Dr. Soetomo Surabaya. The FTIR characterization was carried out with ellagic acid powder, bovine bone xenograft powder, and combined-sample powder with a ratio of 50:50.

An FTIR spectrometer Varian 800 of Scimitar Series was used to measure and analyze ellagic acid, calcium phosphate and combination spectra at a wavelength range of 400-4,000 cm^{-1} and the precision of 4 cm^{-1} using the RESOLUTION software. The samples were prepared by combining the powders with KBr and pressing the pellet. This sample preparation technique had a few complications and required professional experience to acquire a good quality range in daily work. Special factors i.e., pellet thickness, particle dispersion, ensuring vacuum state during the pressing, pressure influence, and ion exchange must be considered when performing an invariable sample analysis using the KBr method. There should be a 2-10% material concentration from the total weight of a prepared KBr pellet. To prepare a 300g KBr pellet, a sample of 1 to 5mg and a pellet size of 13 mm was required.

The size of the powder grain size was ~150 ┼m. The analyzed samples were crushed into a powder and thoroughly combined with the KBr powder. “Pulverisette 23”, a powder mixer, was used for crushing and blending the powder. The prepared powder was then added to a specific SPECAC (d = 13 mm) mould, and a uniaxial press (required pressure is ~5.103 kg/cm2, pressing time 1 min) was applied to reach the required pressure. Additionally, KBr created wide water bands in the spectrum as they attracted water molecules within the environment. As a result, they may be difficult to or impossible to study. Therefore, KBr powder should be of the highest assays, and the Riedel-de-Haen KBr (Lot 51520) brand was used with an assay of 99.5-100.5%. Typically, absorption bands of the main impurities in the KBr were OH-groups and H2O molecules (3500 cm^{-1} and 1630 cm^{-1}), NO_2 (1390 cm-1), and SO_4^{2-} (1160-1140 cm^{-1}). As the powder was hygroscopic, KBr powder was dried at 105°C and kept in special hermetic containers.[8]

No.	Samples	Information
1.	Ellagic acid powder	Ellagic acid dihydrate, 97%
2.	Bovine bone xenograft powder	Crystalline calcium phosphate
3.	Sample combination 50:50	Ellagic acid dihydrate, 97%

Table 1. Sample treatment for FTIR characterization.

The FTIR characterization aimed to identify the existing molecular bonds and analyze them on each identified functional group. The analysis conducted refers to bond stretching and bond bending. The FTIR resulted in the form of graphs or curves of relative transmittance (%) to wave number (cm^{-1}). Visible light consists of several different ranges of electromagnetic frequency which frequency had a different color. Infrared radiation also contained several frequency ranges which were invisible. Measurements were carried out in the mid-infrared region in the infrared spectrum at a wavelength of 2.5-50 m or a wave number of 4,000-200 cm^{-1}. The resulting energy from the radiation would create vibrations in the molecules. The infrared absorption band was notably distinctive and specific for each type of chemical bond or functional group. This method was particularly helpful in identifying organic and organometallic compounds.

Commonly used light sources are tungsten lamps, Narnst glowers, or glowbars. Infrared dispersion spectrophotometer used a monochromator to select wavelengths. In the situation that a specific infrared radiation frequency passed on an organic compound sample, the compound will absorb the frequency of the radiation. A detector on the other side of the compound would recognize frequencies passed through the sample and not absorbed by the compound. The number of frequencies passed through the compound (not absorbed) would be computed as a percentage of transmittance. If there is 100% transmittance, none of the IR frequencies will be absorbed by the compound. However, this condition has never happened. There was always a small amount of this frequency absorbed and giving a transmittance of as much as 95%. A

transmittance of 5% means that the compound absorbed almost all of the transmitted frequency. This very high absorption could provide important information about the bonds in this compound.

The resulting spectrum in a graph showed the percentage of transmittance that varied at each frequency of infrared radiation. The unit of frequency used on the horizontal line (axis) was expressed in wavelength. Each frequency of light, including infrared radiation, had a specific energy. If the frequency of light absorbed by the compound is found during the investigation, the energy will be transferred to the compound. The amount of energy absorbed by the compound would affect the condition of the molecules of the compound. The energy of infrared radiation was related to the energy required for the vibration of a bond.

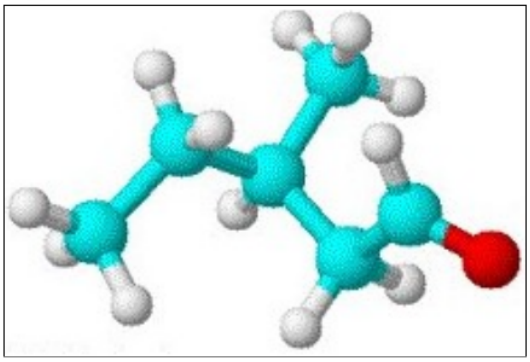

Figure 1. Ellagic acid powder (Five-carbon chain; IUPAC name = 3-Methylpentanal)

The common names for aldehydes are derived from the names of the corresponding carboxylic acids by replacing the -ic with -aldehyde. The IUPAC naming is similar to other hydrocarbons with the longest chain carrying the -C(O)H group considered as the parent structure because of their ability to form hydrogen bonds (the lower molecular weight aldehydes) and to be appreciably soluble in water.

Figure 2 shows how amides have quite high boiling points because they are capable of strong intermolecular interactions.

In the laboratory, amides were made as a result of the reaction between ammonia and acid chlorides or acid anhydrides. Industrially, they were often prepared through the heating of carboxylic acids' ammonium salts.

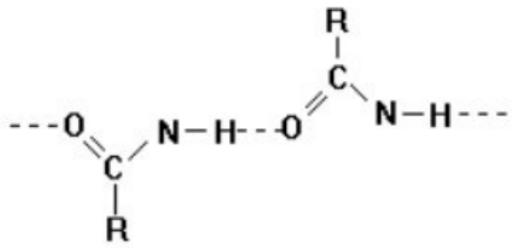

Figure 2. Bovine bone xenograft powder (Amida).

Results

The FTIR analysis of the ellagic acid samples showed that the expected range for the carbonyl absorption in alkyl aldehydes was 1,740-1,730 cm^{-1}. The ketones' case is that the vapor phase spectra are shifted to higher frequencies (about 10-15 cm^{-1}). Unsaturated aldehydes with the double bond positioned in the alpha-beta position absorbed approximately 1,705-1,685 cm^{-1}. Aldehydes conjugated with aromatic rings were absorbed in the 1,710-1,690 cm^{-1} regions. Halogens next to the aldehyde carbon showed an identical shift to higher frequencies for ketones. The aliphatic C-H stretching vibrations were highly characteristic and occurred between 2,900 and 2,700 cm^{-1}. The aliphatic C-H stretching was very characteristic and occurred between 2,900 and 2,700 cm^{-1} as shown in Figure 3.

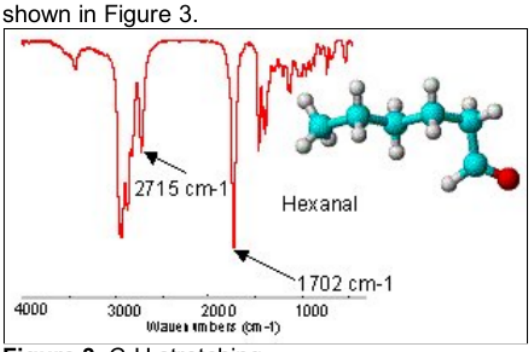

Figure 3. C-H stretching

The results showed the absorbance of 1,053 cm^{-1} wave indicating that there was a CH bending functional group (see Figure 4). The absorbance of 1,110 cm^{-1} wave illustrated that there was a CO alcohol functional group. The absorbance of 1,396 cm^{-1} wave showed that there was a bending CH_3 functional group, and

that of 1,446 cm^{-1} waves indicated that there was a CH_2 bending functional group. Moreover, at the absorbance of 1,446 cm^{-1} wave, a bending function group of 1,693 cm^{-1} wave suggested that the compound had the functional group C=O ketones and aldehydes. At the absorbance of 3,070 cm^{-1} wave, there was also an OH functional group of H bond. Finally, the absorbance of 3,475 cm^{-1} wave resulted in strengthened vibration (overtune).

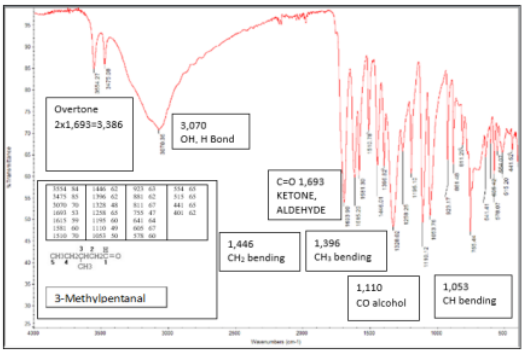

Figure 4. FTIR analysis of the ellagic acid

No	Spectral	Primary	Secondary	Tertiary
1.	NH stretching	Near 3,350-3,180 cm^{-1}	3,320-3,070 cm^{-1} (trans and cis)	
2.	CO (amide I)	Near 1,650 cm^{-1}	1,680-1,630 cm^{-1}	1,670-1,630 cm^{-1}
3.	NH_2 def. (amide II)	1,650- 1,620 cm^{-1}	1,570-1,515 cm^{-1}	
4.	Amide III		Near 1,270 cm^{-1}	

Table 2. FTIR analysis of the bovine bone xenograft samples.

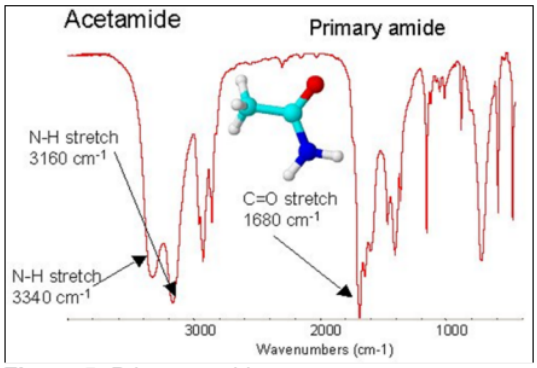

Figure 5. Primary amide.

Figures 5 and 6 show that the amide functional group merges the features of amines and ketones due to it having both the N-H bond and the C=O bond. Therefore, amides showed a strong, somewhat broad band at the left end of the spectrum, at the range of 3,100 and 3,500 cm^{-1} for the N-H stretch. Additionally, they also showed the stake-shaped band in the middle of the spectrum at approximately 1,710 cm^{-1} for the C=O stretch. With amines, primary amides showed two spikes, while secondary amides showed just one spike.

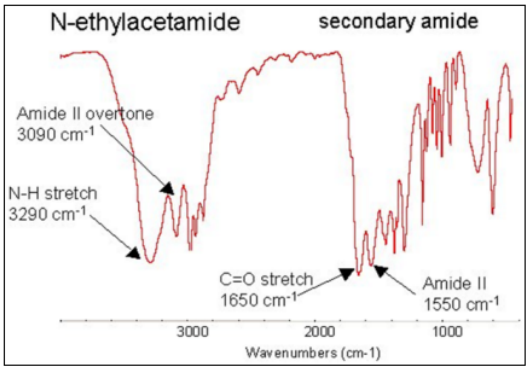

Figure 6. Secondary amide.

Inorganic phosphates, however, had a very characteristic spectrum. There are two strong bands of around 1000 cm^{-1} (figure 7 Potassium phosphate) and 550 cm^{-1} (figure 8 Silver phosphate). There are also usually water bands of around 3400 cm^{-1} and 1640 cm^{-1}. The result of the enlargement is the FTIR analysis of the bovine bone xenograft (see Figure 9).

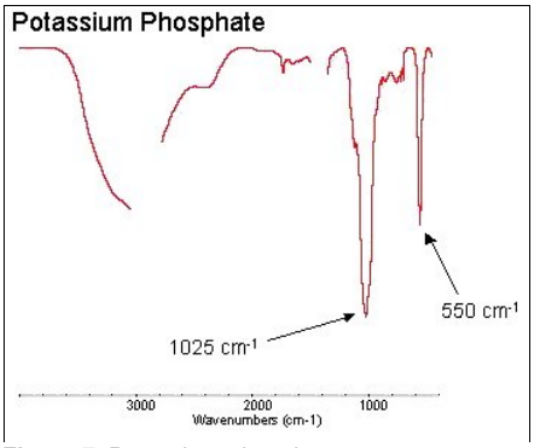

Figure 7. Potassium phosphate

Figure 9 shows the absorbance of 1,007 cm^{-1} wave has a CH2 bending functional group, and the absorbance of 1,400 cm^{-1} wave had a CH bending functional group. At the absorbance of 1,629 cm^{-1} wave, there was a C=C stretching functional group, and at 2,358 cm^{-1} waves, there was a C=C stretching function group. Finally, the absorbance of 3,254 cm^{-1} resulted in a functional group O=H stretching.

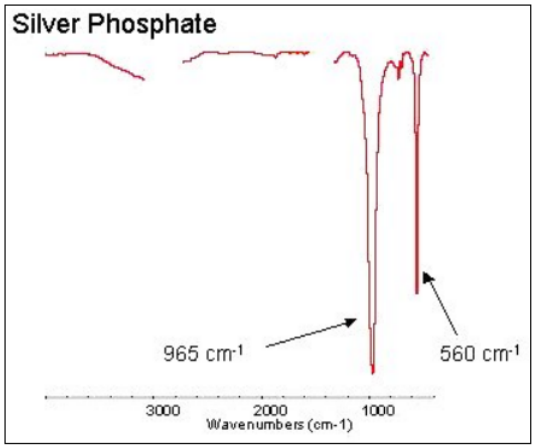

Figure 8. Silver phosphate

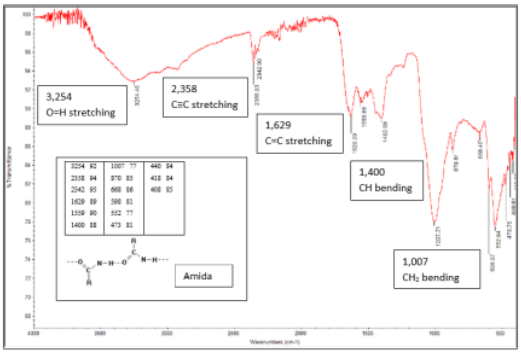

Figure 9. FTIR analysis of the bovine bone xenograft.

Figure 10 shows that at the absorbance of 1,053 cm^{-1} wave, there was a CH_2 bending functional group, and the absorbance of 1,110 cm^{-1} wave had a CH bending functional group. While the absorbance of 1,331 cm^{-1} wave resulted in a CH bending functional group. The result also showed that the absorbance of 1,693 cm^{-1} wave resulted in a C=O stretching function group, and at the absorbance of 3,070 **cm^{-1}** wave, there was a OH stretching functional group. Finally, both the absorbance of 3,474 and 3,554 cm^{-1} wave showed OH stretching functional groups.

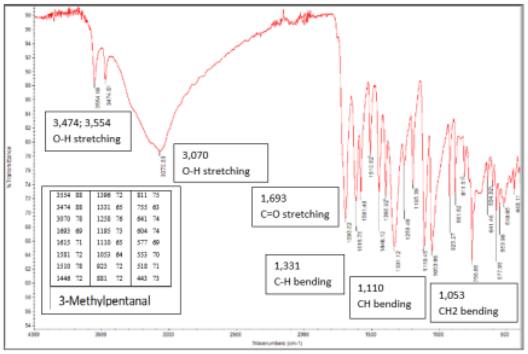

Figure 10. FTIR analysis of the combination of ellagic acid and bovine bone xenograft.

Discussion

The FTIR characterization in this study aimed to identify the molecular bonds in ellagic acid, calcium phosphate, and a combination of both. Based on the results of FTIR characterization, the combined protein was shown by the OH stretching functional group (3,554 cm^{-1}), the OH stretching functional group (3,070 cm^{-1}), the C=O stretching functional group (1,693 cm^{-1}), the CH bending functional group (1,110 cm^{-1}), and the CH2 bending functional group (1,053 cm^{-1}). The different combination between the ellagic acid in C-H bending functional group (1,331 cm^{-1}) and calcium phosphate in C-H bending functional group (1,328 cm^{-1}) showed that ellagic acid was not lost when combined with calcium phosphate, and the compound in ellagic acid was still strong.

The identification of each specific bond absorption of each functional group is the basis for the interpretation of the infrared spectrum. For example, O-H stretching gave a strong absorption band in 3,554 cm^{-1}, and an absorption in 3,070 cm^{-1} is a compound class of alcohol. The O-H bond in alcohol absorbs at a higher wavelength than an acid between 3,230-3,550 cm^{-1}. This absorption is at an even greater wavelength if the alcohol does not contain hydrogen bonds in the gaseous state. The absorption of the C-H bond is slightly below

3,000 cm^{-1}, and the absorption of C-O bond is in the region of 1,000 and 1,100 cm^{-1} wave.[9]

Bonding to the carbonyl group, C=O stretching (1693 cm^{-1}) provided a very useful absorption in 1,680-1,750 cm^{-1} wave of a class of compounds of conjugated acids. The position of the absorption band may vary slightly based on the types of compounds. Another important type to find is the O-H. This bond absorbed wave at different positions depending on the environment. This bond is very easy to identify as an acid because it produces a wide signal in the 2,500-3,300 cm^{-1} region.[10]

Carbon-carbon bonds had absorption in a large range of wave numbers in the fingerprint region; thus, it is very difficult to interpret carbon-carbon bonds from the infrared spectrum. The bending C-H bonds (1,331 cm^{-1} and 1,110 cm^{-1}) were carbon-hydrogen bonds. The carbon-oxygen single bond also has an absorption in the fingerprint region that varies between wavelength in 1,000 and 1,300 cm^{-1}, depending on the molecule.[11]

Each signal occurs due to the absorption of energy at a certain frequency from infrared radiation, resulting in bond vibrations within the molecule. Multiple signals are very easy to use in identifying a particular type of bond in a molecule. In this study, the bending CH2 bond (1,053 cm^{-1}) region to the right of the diagram (from 1,500 to 500 cm^{-1}) usually contained very complex absorbent forms as all types of molecular bending vibrations absorb in this region. This area is called the fingerprint area. It is very difficult to analyze the type of bond in this region. The most important use of the fingerprint region is that every compound gives a differing pattern in this area. For unknown compounds, the infrared spectrum can only be used to see the types of bonds or functional groups present in the compounds. Infrared spectra can only be used for identification if there is a comparison compound spectrum which is observed under identical conditions by contrasting its fingerprint area.[12]

Pomegranates' antioxidant and anti-inflammatory activity occurred because of its extremely high polyphenol content. Based on a systematic literature review by Fatmawati *et al.* the fruit and skin of *Punica granatum* Linn. and its polyphenol content such as ellagic acid, urolithin A, and rosmarinic acid are able to inhibit the proinflammatory cytokine TNF-α. The polyphenols contained in pomegranate can suppress the production of p38-MAPK and NF-kB which are associated with the formation of TNF-. Thus, the contents in the pomegranate can reduce TNF-α levels and reduce inflammation.[13]

In producing materials that can promote bone tissue regeneration, the combination of the two ingredients is synergistic to provide an optimal function. The combination of ellagic acid and bovine bone xenograft can provide a synergistic effect on the process of new bone growth along with bone formation. Bovine bone xenograft is osteoconductive, and such characteristic is the nature of the bone graft matrix to support the attachment of bone-forming cells[4], while ellagic acid is osteo inductive and thus stimulates the differentiation of osteoprogenitor cells into osteoblast cells.[3] Based on the results from the FTIR analysis, the bovine bone xenograft contained crystalline calcium phosphate.

Calcium phosphate presents as an odorless and tasteless white amorphous or crystalline powder. In ethanol and acetic acid, it is insoluble. However, it is soluble in dilute nitric acid and hydrochloric acid. It will partially dissolve in water. It can be found in ground, teeth, milk, and bones.[14] Apatite, a mineral rock, is an impure and complex form of calcium phosphate and yields tribasic calcium phosphate. An appetite is a type of phosphorite containing calcium phosphate along with other compounds.[15,16] Calcium phosphates are among some of the most significant materials in dentistry, medicine, industry, geology, and biology. The functions, formation, and applications of calcium phosphates depend on their stability, solubility, composition, and structure.[17] Animals' hard tissue is mainly made up of crystalline calcium phosphate. In vitro small changes in the reaction conditions have an effect on the species of calcium phosphate that is formed, while in vivo distinct types of crystalline calcium phosphate are shaped in a well-controlled spatiotemporal-dependent state. A range of proteins play a role in hard-tissue formation.[18,19] The results of the identification of functional groups in the combination of ellagic acid and calcium phosphate showed that there are different contents of functional groups that could be identified. The biggest possibility after mixing is that the ellagic acid compound does not disappear due to its strength when mixed with

bovine bone xenograft.

Conclusions

The combination of calcium phosphate and ellagic acid contain different functional groups. When combined, the compounds are still strong in ellagic acid.

Acknowledgements

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Declaration of Interest

The authors report no conflict of interest.

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