Glucosamine production from palmyrah (*Borassus flabellifer* L.) seeds (a study of precursor type and concentration)

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**Abstract.** Glucosamine is an amino-sugar compound that is a precursor in synthesis repairment of cartilage and joints in human body. Glucosamine can be produced in the body as well as obtained from animal and plant sources. One of the plant sources that contain glucosamine is the seed of palmyrah. To obtain glucosamine from palmyrah seeds is required extraction process. The addition of ammonium salt precursor to the extraction stage could increase the production of glucosamine. The aim of this research was to determine the influence of the type and concentration of ammonium salt precursor to the glucosamine content from seeds of palmyrah fruit. This research used nested design with two factors, consisting of type and concentration of precursor. The main factor of precursor type (as first factor) consisting of three levels, namely NH₄Cl, (NH₄)₂CO₃, and (NH₄)₂SO₄, although nested factor of precursor concentration (as second factor) consisting of two levels. The glucosamine content was tested by spectrophotometry analysis. The effect of treatment was analyzed using analysis of variance and was continued with the Least Significant Difference test. Glucosamine was tested qualitatively by Thin Layer Chromatography. The results showed that the best treatment of precursor types were found in the addition of NH₄Cl 4.5 M; (NH₄)₂CO₃ 1.5 M; and (NH₄)₂SO₄ 4 M with glucosamine content 1418.5; 1263.5 and 1078.5 ppm and yield 0.450; 0.380; and 0.627% respectively. The qualitative test using TLC showed all samples of extracts have similarities of retention factor with glucosamine standard.

1. **Introduction**

Palmyrah (*Borassus flabellifer* L.) is a type of palm plant that can grow in dry climates, one of which is in Tuban Regency. According to data from the Central Bureau of Statistics in 2013 [1], Tuban Regency is the largest producing palmyrah in East Java Province. In the period 2009-2012, with an area of 1206 hectares, the average production of palmyrah produced was 4.8 tons per hectare per year. Palmyrah fruit contains various compounds that are good for health in addition to utilization in the medical field. Kernel in palmyrah fruit contains free sugar like sucrose (0.38%), fructose (1.46%), and glucose (3.21%) [2]. In addition, the extract of palmyrah seed flour has a carbohydrate isomer structure called tetraglycosides pirostane, which contains residues in the form of three α-rhamnosyl and one β-pyranosil. Comparison with known spectral data showed that the β-pyranosyl is likely to be a β-glucosaminosyl (glucosamine) which is probably in position 6 of glucose in one compound and most likely to be in the position 3 of glucose in the other [3].
Glucosamine or known as amino sugar is a monosaccharide class compound. Glucosamine can be produced through plant extracts, such as corn [4] and chicory roots [5]. In the human body, glucosamine acts as a cartilage-forming compound incorporated in the connective tissue. Lack of glucosamine compounds can cause osteoarthritis (arthritis). For this reason, additional intake of glucosamine is needed which serves to reduce or repair cartilage damage and also increase cartilage production in the body [6]. Seeing the high potency for glucosamine that can be used as a drug for osteoarthritis, now there are many developed glucosamine production through extraction from various kinds of raw materials, especially in plants.

Production of glucosamine can be formed through reactions between compounds derived from carbon sources (D-glucose, D-fructose, maltose, sucrose, or rice starch) and compounds derived from nitrogen sources (ammonium salts, sodium nitrate, monosodium glutamate, or yeast extracts) [7]. Chemical reaction of glucosamine formation can be carried out by replacing OH- groups in glucose, with amine groups (NH2-) obtained from ammonium salt solutions as precursors [8]. According to Brown et al. [9], amine groups can act as good nucleophiles (electron carriers). Precursor solutions have a variety of negative ion sources, which can affect the changes hydroxyl groups with amines because the negative ion size varies. According to Brown et al. [10], large ionic diameter indicates that the valence electrons possess are easily released. It is interesting to study the effect of different types of precursors on glucosamine formation.

Therefore, this research studied the effect of precursor types of several types of ammonium salts and its concentration added to the glucosamine formation reaction from pieces of seeds extracted by maceration, so as to produce the best quality glucosamine.

2. Materials and Methods
2.1. Materials
The raw materials palmyrah seeds (*Borassus flabellifer* L.) used in the study were approximately 3 weeks old. These obtained from sellers in the Malang, East Java, which is supplied directly from Tuban Regency, East Java. Supporting materials include ammonium chloride, ammonium sulfate, ammonium carbonate, aquades (pH = 7), glacial acetic acid, reference standard of glucosamine hydrochloride SIGMA-ALDRICH, butanol, and TLC plates.

2.2. Method
2.2.1. Experimental design
The experimental design of this research was Nested Design method, with ammonium salts precursor types as the main factor, and precursor concentration as the nested factor. The main factors consist of 3 levels, namely ammonium sulphate, ammonium carbonate, and ammonium chloride. The nested factor consists of 2 levels in every ammonium salts precursor types. They were ammonium sulfate precursor (concentration 3.5 M and 4 M), ammonium carbonate (concentration 1.5 M and 2 M); and ammonium chloride (concentration 4.5 M and 5 M). Each combination treatment was repeated 3 times, resulting in 18 experimental units (see Table 1).

2.2.2. Production of glucosamine
Production of glucosamine in this research was conducted according Courtois [5] with modification. The seeds of palmyrah fruit were peeled and washed. Palmyrah seeds were weighed as much as 100 grams. The palmyrah seeds were diced in the size of 0.5 x 0.5 x 0.5 cm and then were soaked in 50 ml of various types of ammonium salt precursors. The concentrations used are ammonium sulfate (3.5 M and 4 M); ammonium carbonate (1.5 M and 2 M); and ammonium chloride (4.5 M and 5 M). It soaked for 12 hours in the oven at 70˚C of temperature. After being soaked, the pieces of the seeds were filtered and dried using an oven at 55˚C for 24 hours. Then, the slices of palmyrah seeds were extracted by maceration (room temperature) with 100 ml distilled water for 3 hours and filtered. The resulting filtrate was then dried using a vacuum oven at 60˚C for 50 hours, with a pressure of 20 mbar. These step produced dried extract of crude glucosamine.
Table 1. Experimental design.

| Main factor: Ammonium salts precursor types | Nested factor: Concentration (M) |
|-------------------------------------------|---------------------------------|
| NH₄Cl                                     | 4.5                             |
|                                           | 5.0                             |
| (NH₄)₂CO₃                                 | 1.5                             |
|                                           | 2.0                             |
| (NH₄)₂SO₄                                 | 3.5                             |
|                                           | 4.0                             |

2.2.3. Glucosamine content analysis

Spectrophotometric method was used to analyze the glucosamine content [15, 16]. For calibration curve, a series of dilution of glucosamine hydrochloride as reference standard were prepared in 0.01% acetic acid solution to obtain the concentrations 10, 20, 30, 40, 50, 60 and 70 ppm. The absorbance was measured on UV/Vis spectrophotometer at 190-240 nm against blank (according maximum of wave length was obtained). Maximum wave length of this research is 190 nm. For quantification the glucosamine content of extracts, 0.25 g of extract was diluted to 10 ml acetic acid 0.01% and the absorbance was measured at 190 nm of UV/Vis spectrophotometer. The glucosamine content was calculated from calibration curve.

2.2.4. Glucosamine yield

To determine glucosamine yield, was needed data from glucosamine content. The procedure of determining glucosamine yield as followed: weight of extracts was multiplied by glucosamine content so to get weight of glucosamine. The yield was obtained by comparing the weight of glucosamine with the initial weight of palmyrah seed being used and multiplied by 100%.

2.2.5. Thin layer chromatography (TLC)

The best treatment from nested factor was qualitatively tested using TLC. In this method, required alumina as stationary phase and solvent (mobile phase) consist of buthanol: acetic acid: distilled water with ratio of 37: 25: 9. Spot of sample and reference standard of glucosamine hydrochloride were applied to the stationary phase plate and developed in the chamber. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent. Rf value can be used to identify compounds due to their uniqueness to each compound. To determine glucosamine in the extract required Rf value of glucosamine hydrochloride to be compared with Rf value of sample. Rf value each spot can be measured by dividing both of distance migrate of spot sample by solvent. The same Rf indicates the same compound [17].

2.3. Statistical analysis

Observation data were analysed by the Nested Design method, where the main factors were analyzed using ANOVA and continued with the Least Significant Difference (LSD) test with a 5% confidence interval, while the nested factors were analyzed using ANNOVA without continuing with LSD test.

3. Results and Discussion

3.1. Glucosamine content

The types of precursors (ammonium sulfate, ammonium carbonate, and ammonium chloride) have a significant effect on glucosamine content (Table 2). Based on it, it is known that the addition of ammonium sulfate salt precursors to palmyrah seed produce the highest glucosamine content. Ammonium sulphate is composed from NH₄⁺ and SO₄²⁻ ions, which is the SO₄²⁻ ions (258 pm) have a longer diameter of ions compared to ions Cl⁻ (184 pm) and CO₃²⁻(178 pm). The longer the diameter of the negative ions, the smaller the competition with amine ions (NH₂⁻) in binding glucose to replace ion...
hydroxyl (OH ) that have diameter 133 pm. The NH₃⁺ groups have the smallest diameter of ions (130 pm). This causes NH₃ groups can easily bind into glucose compounds if compare with SO₄²⁻, Cl⁻ and CO₃²⁻ ions. NH₃⁺ groups can bind easily when negative ions (nucleophile) as competitor have longer diameter. That is why, the highest content of glucosamine obtained from precursor (NH₄)₂SO₄.

The amount of glucosamine content is also influenced by the number of moles and particles in the amine ion (NH₄⁺). According to Chang [11], the large number of particles causes increasing collisions of ions, so that the resulting product also increases. The greater the number of moles and the number of amine ion particles contained in ammonium salts, the higher the particle collision frequency between the amine group and glucose, thus affecting the high amount of glucosamine produced.

| Table 2. Glucosamine content. |
|-------------------------------|
| Ammonium salts precursor types | Glucosamine content (ppm) |
| NH₄Cl                          | 876.83⁹                     |
| (NH₄)₂CO₃                     | 1003.50⁹b                   |
| (NH₄)₂SO₄                     | 1086.00⁹a                   |

Note: Numbers followed by a different notation indicates the difference between treatments (LSD 5%= 207.77)

| Table 3. Glucosamine content in different precursor concentration. |
|-------------------------------------------------------------------|
| Ammonium salts precursor types | Concentration (M) | Glucosamine content (ppm) |
| NH₄Cl                          | 4.5              | 1418.5⁶                     |
|                                | 5.0              | 588.5⁶b                   |
|                                | 1.5              | 1263.5⁶m                   |
| (NH₄)₂CO₃                     | 2.0              | 490.2⁶                   |
|                                | 3.5              | 1093.5⁶                |
| (NH₄)₂SO₄                     | 4.0              | 1078.5⁶                   |

Note: Numbers followed by a different notation indicates the difference between treatments (α=0.05)

The addition of the amount of concentration in each precursor also had a significant effect on the amount of glucosamine content. Table 3 shows that higher concentrations of precursors actually reduce glucosamine levels. Decreasing in glucosamine levels is strongly influenced by the reaction rate between glucose and ammonium salts. In addition, more and more ions can also cause increasing in the frequency of collisions between ions. It is suspected too high a concentration of precursors can cause the breaking of ionic bonds between glucose and amines, so that the resulting glucosamine levels decrease.

3.2. Yield
Precursor types give a significant effect on glucosamine yield (α = 0.05). Based on Table 4, ammonium sulphate precursor produces the highest glucosamine yield. The yield of glucosamine is directly proportional to the content of glucosamine extract. According to Dyah and Simon [12], high yields indicate that the extraction process can produce pure extract (contains a little impurity). So that it can be said that the glucosamine extract from the palmyrah seeds has high quality.

The concentrations of precursors did not give a different effect on glucosamine yield (Table 5). The yield is influenced by components lost during the processing process [13]. This condition is thought to have originated from the immersion process. The immersion process results in the binding between glucose and the amine group in the ammonium salt. This is because each type of ammonium salt has a concentration limit of each in binding to glucose, so that in the immersion process, the chip only
absorbs a small portion of ammonium salt solution in accordance with its capacity. In addition, the yield value is also strongly influenced by the age of harvest from the raw material used.

### Table 4. Glucosamine yield.

| Ammonium salts precursor types | Yield (%) |
|-------------------------------|-----------|
| NH₄Cl                         | 0.27ᵇ     |
| (NH₄)₂CO₃                     | 0.22ᵇ     |
| (NH₄)₂SO₄                     | 0.67ᵇ     |

Note: Numbers followed by a different notation indicates the difference between treatments (LSD 5% = 0.24)

### Table 5. Glucosamine yield in different precursor concentration.

| Ammonium salts precursor types | Concentration (M) | Yield (%) |
|-------------------------------|-------------------|-----------|
| NH₄Cl                         | 4.5               | 0.45⁰ᵃ    |
|                               | 5.0               | 0.24⁰ᵃ    |
| (NH₄)₂CO₃                     | 1.5               | 0.38⁰ᵐ    |
|                               | 2.0               | 0.18⁰ᵐ    |
| (NH₄)₂SO₄                     | 3.5               | 0.68⁵ˣ    |
|                               | 4.0               | 0.62⁵ˣ    |

Note: Numbers followed by a different notation indicates the difference between treatments (α=0.05)

3.3. Thin layer chromatography

TLC analysis was only carried out on samples with the best treatment, i.e. the addition of ammonium chloride salt precursors with a concentration of 4.5 M, ammonium carbonate salt with a concentration of 1.5 M, and ammonium sulfate salt with a concentration of 4 M. TLC test is intended to find out the pattern resulting from the separation of glucosamine compounds contained in the sample. In the TLC test of glucosamine extract, is used alumina as the stationary phase. Alumina is a polar stationary phase that can separate amine compounds. The mobile phase (eluent) used in the separation of glucosamine is made of butanol: acetic acid: distilled water (37: 25: 9). The eluent is polar, with a polarity index value of 0.643. The high polarity index value (near 1.0) shows the higher the level of polarity.

Based on the TLC test (Figure 1), the result of Rf value on glucosamine standard was 0.855, Rf value on extract from glucosamine ammonium chloride, ammonium carbonate and ammonium sulfate were 0.839; 0.871, and 0.887, respectively. The distance migrated of spots on the TLC plate almost the same. Rf value results show that the glucosamine standard and all of glucosamine extracts have the same characteristics because produce the same Rf value. The same Rf value between standard and sample indicate the same compound. So, it was proved that all of extract contain glucosamine.

According to TLC test result (Figure 1), sample a (standard glucosamine) and sample d (Ammonium sulfate) produce one spot with almost the same size in their zones of migration or movement. According to Harwood and Ian [14], pure sample only produced one spot in the zone TLC plate. Therefore, it can be said that sample from 4 M ammonium sulfate turn to contain pure glucosamine. On the other hand, sample b (ammonium chloride) and sample c (ammonium carbonate) produce more than one spot. It was indicate that the more spots produced in the zone of migration or movement by the sample in the TLC plate, the lower level of purity. Therefore, it can be said that extract samples from 4 M ammonium chloride and 1.5 M ammonium carbonate still contain impurities.
Figure 1. TLC result of glucosamine samples and standard in normal room light (1) and UV light (2). Spot of glucosamine standard (a), spot of glucosamine extract from ammonium chloride precursor (b), spot of glucosamine extract from ammonium carbonate precursor (c), spot of glucosamine extract from ammonium sulphate precursor (d), end spot movement of glucosamine standard (a’), end spot movement of glucosamine extract from ammonium chloride precursor (b’), end spot movement of glucosamine extract from ammonium carbonate precursor (c’), end spot movement of glucosamine extract from ammonium sulfate precursor (d’).

4. Conclusion
The precursor types and concentration affected to glucosamine's yield and content. The best treatments of glucosamine palmyrah seeds were obtained from ammonium chloride 4.5 M, ammonium carbonate 1.5 M and ammonium sulfate 4 M. The characteristic glucosamine from precursor ammonium chloride 4.5 M as follow: 1418.5 ppm of content and 0.450% of yield. The characteristic glucosamine from precursor ammonium carbonate 1.5 M as follow: 1263.5 ppm of content and 0.380% of yield. The characteristic glucosamine from precursor ammonium sulfate 4 M as follow: 1078.5 ppm of content and 0.627% of yield. Qualitatively, glucosamine extracts from 4 M ammonium sulfate salts are similar closer to glucosamine standards, the Rf value is 0.887.

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