Identification of galactogogues in *Gliricidia maculata*

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Abstract. Galactogogues are substances used by periparturient dairy cows for boosting milk production and preventing negative energy balance. A long-term use of commercial galactogogues has a toxic effect to normal health status of both human and animals. The study was conducted to identify herbal galactogogues from bioactive compounds of *Gliricidia maculata* ethanol extract. The samples were harvested randomly from edible portion of plants at optimal age (80 days). Lyophilization was carried out at 55°C for 3 days followed with pulverization. The bioactive compounds were extracted using ultrasonic assisted extraction method and identified by the GCMS-QP2010S SHIMADZU instrument with Rtx 5 MS column. Spectra produced by MS was translated into chemical structures and data were presented in form of retention time (minutes), peak area (%), molecular formula (CxHyOz), molecular weight and the name of bioactive compound based on the similarity index of the GCMS library. As the result, there were five major galactogogues of *Gliricidia maculate* namely phytol, 1-Octadecyne, 2,6,10,15,19,23-hexamethyl-Squalene, n-Eicosane and 4-Hexadecen-6-yn which identified in this study. Since this study was the first work reporting galactogogues derived from *Gliricidia maculate*, therefore application of its potential could be implemented in the future study of dairy cattle feeding practices.

1. Introduction
Optimal milk production determines the success of dairy business. The unbalanced between high milk production and nutrient requirements at the beginning of lactation causing negative energy balance (NEB), and resulted in many metabolic disorders and other diseases. To prevent NEB, number of strategy could be be used in the early period of lactation as well as increasing milk production [1, 2]. The addition of feed additives may have a potential effect to improve health status, decreasing of early post-partum diseases and improving of milk yield and quality [3].

Galactogogues are substance used to initiating, maintaining, and augmenting of adequate milk production. Its could be in form of synthetic, plant-derived or endogenous products. They act through exerting an influence on adreno-hypothalamo-hypophyseal-gonadal axis by blocking hypothalamic dopaminergic receptors or by inhibiting dopamine producing neurons. This substance increase prolactin secretion by antagonizing dopamine receptors [4, 5].

Currently, the use of herbal medicines has increased including the use of herbal galactogogues. The increased of herbal medicine application was encouraged by organic product trend and the growing evidences of its safety and efficacy [6]. There are numerous references about herbal galactogogues,
however, they are mainly based on empirical traditions and human studies. For that, in this study a potential source of herbal galactogogues derived from *Gliricidia maculata* was identified for its bioactive components. Further, this herbal source could be used and/or applied in dairy feeding practices.

2. Material and methods

2.1. Material

The materials of this study were the edible portion of *Gliricidia maculata* plants and 96% ethyl alcohol (C₂H₅OH, Merck, pro analysis (PA)). The instrument used in this research are Explorer® Semi-Micro Ohaus E12140 Analytical Balances, Sanyo Drying Oven MOV-112, Foss Tecator CyclotecTM 1093 Sample Mill, Kodo Ultrasonic Bath JAC-2010, Eppendorf 5417C Centrifuge, and SHIMADZU GCMS-QP2010S Rtx 5 MS.

2.2. Methods

The edible portion of *Gliricidia maculata* plants, samples, was collected from Laboratory of Forage and Pasture Science, Faculty of Animal Science Universitas Gadjah Mada. Samples were harvested at optimal age (80 days) in afternoon time (15:00 to 16:00 h), followed with measuring its fresh weight (as fed) using Explorer® Semi-Micro Ohaus E12140 Analytical Balances for getting a fresh weight. Lyophilization was carried out using Sanyo Drying Oven MOV-112 at 55°C for 3 days and pulveration was done by the Foss Tecator Cyclotec™ 1093 Sample Mill with 300 mesh (1 mm screen).

Bioactive compounds were extracted by ultrasonic assisted extraction method using Kodo Ultrasonic Bath JAC-2010 by dissolving 2 grams of sample into 96% ethyl alcohol (C₂H₅OH) PA, and the vibration frequency was set in 20 to 40 kHz for 90 minutes. To separated the solvent and solid, centrifugation at 2000 rpm for 5 minutes in an Eppendorf 5417C Centrifuge was applied to the sample. Then, identification of bioactive compounds was done by SHIMADZU GCMS-QP2010S Rtx 5MS instrument (low polarity, 30 meters length, 0.25 mm internal diameter, 0.25 µm of cross bond diphenyl dimethyl polysiloxane wall coated open tubular, Helium (He) carrier gas, 70 e.v. of ionizing energy and 300°C injection temperature). The chromatograms produced by gas chromatography (GC) were identified by mass spectroscopy (MS), then the spectrum translated into chemical structures and the bioactive compound name was identified based on the similarity index of GCMS library. In the current study, the determination of galactogogues properties were based on the references studied.

The result of this study will be described according to the bioactive compounds which found in *G. maculata* as potential of herb galaktogogues source. The bioactive compounds profile was then presented in table including retention time (minutes), peak area (%), molecular formula (CxHyOz), molecular weight, the bioactive compound name and its potential effects.

3. Results and discussion

*Gliricidia maculata* was the plan which selected as the research material in this study. The reasons where it is usually given to animal as feed ingredients, moreover this plan is easy to grow and found in whole season during the year [7]. To extract the bioactive compound, we select Ethyl alcohol (C₂H₅OH) as solvent due to it, has low toxicity, and tends to be easily separated [8]. Then, the ultrasonic method was a non-destructive and non-invasive, therefore it can be easily adapted to various applications. The advantages of the ultrasonic extraction method were accelerating the extraction process (compared to thermal or conventional method), safer, increases the amount of crude extraction (yield), reduce the operating temperature (for samples that are easily damaged by heating). According to that reasons, it is suitable to be applied to extract the bioactive compounds [9].

The basic principles of separation in the analysis of bioactive compounds was its affinity for the column based on its polarity. Thus, the polar compounds are retained longer in the column than non-polar compounds, according to the rules of like dissolve like in a chemical reaction, so they have a
higher retention time. Retention time was the length of time required by the mobile phase to carry a moving compound after passing through the column to the MS detector, each compound will be break down into form of an ionized fragment (by the 70 e.v. of ionizing energy) to be detect the time and charge ratio for quantify the dominance of the compounds [10]. The separation of compounds in GCMS analysis is also influenced by the boiling point of the compounds, since it is related to the polarity and weight of the molecule. Compounds with a high polarity and molecular weight require more energy to break the intermolecular bonds therefore the boiling point is higher [11].

Figure 1. Chromatogram of Gliricidia maculata ethanol extract

The Gliricidia maculata ethanol extract chromatography using GC instrument produced 32 main peaks (Figure 1), which shows the number of major bioactive compounds detected by MS instrument. All of bioactive compounds appearing in the dominant peaks of chromatogram have a galactogogues properties, direct and or indirect effects. A direct effect means that these compounds directly affect the target organ, the mammary glands, including the development and proliferation of the mammary gland secretory cells (mammogenesis) or increasing prolactin secretion to induce the process of lactogenesis and galactopoiesis.

There were five major bioactive compounds (Bold in Tabel 1) found in Gliricidia maculata ethanol extract, i.e.: phytol (24.07%); 1-Octadecyne (15.19%); 2,6,10,15,19,23-hexamethyl-Squalene (11.40%); n-Eicosane (8.28%); and 4-Hexadecen-6-yne (6.32%). Phytol (C20H40O) was an acyclic alcohol diterpene which can be used as a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1. In ruminants, rumen fermentation of feed ingredients derived from legumes produced a phytol, a chlorphyll constituent, which was then converted to the phytanic acid or 3,7,11,15-tetramethyl hexadecanoic acid (C20H40O2) and stored in fat, and can be used as an energy sources of lactating process in the onset of periparturient dairy cows [11].

Phytanic acid can induced a large amounts of reactive oxygen formation in the mitochondria and also induced the release of Cytochrome C from mitochondria, so it can be used as an immunomodulatory compound [13]. 1-Octadecyne (C18H34) was an alkene, has a long-chain hydrocarbon. 1-Octadecyne, an alpha-olefin, was compatible with oleic acid. In chemical terms, oleic acid is classified as a monounsaturated omega-9 fatty acid. Alkene was easily bounding with oleic acid. May it will synergize with oleic acid to form a long chain unsaturated fatty acid, so it might increase the amount of conjugated linoleic acid in milk yield.

The third major bioactive compounds of Gliricidia maculata ethanol extract was 2,6,10,15,19,23-hexamethyl-Squalene (11.40%). It is an organic compound that serve as antioxidant and was useful for increasing a high-density lipoprotein (HDL). Eicosane (alternative spelling eicosane) is an alkane. Eicosanoids were the signaling molecules made by enzymatic and non-enzymatic oxidation of arachidonic acid or other polyunsaturated fatty acids (PUFAs), which were similar to arachidonic acid, with a length of 20 units of carbon. Eicosanoid was a sub-category of oxylipine, i.e. oxidized fatty acids from carbon units of varying lengths, and were distinguished from other oxylipines by their extraordinary importance as cell signaling molecules. The functions of eicosanoids in various physiological systems and pathological processes such as encourage or inhibit inflammation, allergies, fever, and other immune responses. It also regulates the normal pregnancy and abortion, contribute to
the perception of pain, regulate cell growth, controlling blood pressure, and modulate the regional flow of blood to the tissue. In carrying out this role, eicosanoids most often act as autocrine signaling agents to influence their original cells or as paracrine signaling agents to influence the cells near their original cells. Eicosanoid also able to act as an endocrine agent to control distant cell function. The 4-Hexadecen-6-yne \((\text{C}_{16}\text{H}_{26})\) compound is an immunomodulatory compound [14].

**Table 1.** Profile of *Gliricida maculata* ethanol extract bioactive compounds

| No. | Peak | Retention Time (RT) | Peak Area (%) | Name | Chemical Structure | Similarity Index (SI) | Lacto-gogues Activity | References |
|-----|------|---------------------|----------------|------|--------------------|-----------------------|------------------------|------------|
| 1   | 12,635 | 1.14 | | Azidine; Ethylene imine; Aminomethylene | C\(_5\)H\(_{11}\)N | 80 | - | |
| 2   | 21,449 | 0.42 | | 2-acetyl-ethyl-3-carene | C\(_{12}\)H\(_{20}\)O | 73 | - | |
| 3   | 25,107 | 0.49 | | Fumaric acid | C\(_{10}\)H\(_8\)O\(_4\) | 72 | + | [14, 15] |
| 4   | 28,857 | 0.35 | | Patchouli alcohol | C\(_{16}\)H\(_7\)O | 81 | + | [16] |
| 5   | 31,406 | 0.95 | | Bicyclo(2,2,2)octane-1,4-diol, monoacetate | C\(_{16}\)H\(_{16}\)O\(_1\) | 76 | + | [17] |
| 6   | 32,063 | 0.91 | | 3-Tetradecene | C\(_{14}\)H\(_{28}\) | 91 | + | [18] |
| 7   | 32,228 | 15.19 | | 1-Octadecene | C\(_{18}\)H\(_{34}\) | 90 | + | [19] |
| 8   | 32,346 | 2.42 | | 1-Decene, 8-methyl | C\(_{11}\)H\(_{22}\) | 90 | + | [18] |
| 9   | 32,740 | 2.19 | | 1-Octadecene | C\(_{18}\)H\(_{34}\) | 89 | + | [19] |
| 10  | 33,144 | 5.37 | | 1-Octadecyne | C\(_{18}\)H\(_{34}\) | 90 | + | [19] |
| 11  | 34,127 | 0.92 | | Octadecanoic acid, methyl ester | C\(_{14}\)H\(_{26}\)O\(_2\) | 93 | + | [17] |
| 12  | 34,533 | 0.17 | | 1-Penten-3-ol, 3-methyl; Ethylbutenol | C\(_{4}\)H\(_8\)O | 69 | + | [20] |
| 13  | 34,833 | 0.21 | | 1,2-Benzenedicarboxylic acid, dibutyl ester; Butyl phthalate | C\(_{16}\)H\(_{20}\)O\(_4\) | 70 | + | [18] |
| 14  | 34,925 | 0.57 | | Hexadecanoic acid; Palmitic acid | C\(_{16}\)H\(_{32}\) | 89 | + | [17] |
| 15  | 35,514 | 2.71 | | Hexadecanoic acid, ethyl ester; Ethyl palmitate | C\(_{18}\)H\(_{32}\)O\(_2\) | 93 | + | [17] |
| 16  | 37,111 | 0.58 | | 9,12-Octadecadienoic acid; methyl ester | C\(_{18}\)H\(_{32}\)O\(_2\) | 90 | + | [17] |
| 17  | 37,639 | 1.34 | | 11-Octadecenoic acid; methyl ester | C\(_{18}\)H\(_{32}\)O\(_2\) | 90 | + | [17] |
| 18  | 37,881 | 24.07 | | **Phytol** | C\(_{20}\)H\(_{42}\) | 90 | + | [17, 21]; [20, 22] |
| 19  | 38,133 | 0.03 | | Hexanoic acid, 2-methyl; 2-Methylhexanoic acid | C\(_{10}\)H\(_{18}\)O\(_2\) | 64 | + | [17] |
| 20  | 38,789 | 2.47 | | 9,12-Octadecadienoic acid; methyl ester | C\(_{18}\)H\(_{34}\)O\(_2\) | 89 | + | [17] |
| 21  | 38,912 | 6.32 | | 4-Hexadecen-6-yn | C\(_{18}\)H\(_{32}\) | 85 | + | [23] |
| 22  | 39,025 | 0.40 | | Heptadecenoic acid; ethyl ester; Ethyl n-heptadecanoate | C\(_{19}\)H\(_{36}\)O\(_2\) | 79 | + | [17] |
| 23  | 39,392 | 0.82 | | 2-Hexyl-1-octanol | C\(_{18}\)H\(_{36}\)O\(_2\) | 93 | + | [17] |
| 24  | 41,318 | 0.30 | | Hexadecane; n-Hexadecane; n-Cetane; Isohexadecane | C\(_{16}\)H\(_{34}\) | 87 | + | [22] |
| 25  | 44,717 | 0.80 | | Hexadecane; n-Hexadecane; n-Cetane; Isohexadecane | C\(_{16}\)H\(_{34}\) | 95 | + | [18] |
| 26  | 47,862 | 3.51 | | Eicosane | C\(_{20}\)H\(_{42}\) | 97 | + | [18] |
| 27  | 49,351 | 0.25 | | Pentadecane | C\(_{19}\)H\(_{32}\) | 86 | + | [18] |
| 28  | 49,590 | 11.40 | | 2,6,10,14,18,22-Tetracosahexaene; 2,6,10,15,19,23-hexamethyl-Squalene | C\(_{36}\)H\(_{60}\) | 93 | + | [17] |
| 29  | 50,796 | 8.28 | | Eicosane; n-Eicosane | C\(_{20}\)H\(_{42}\) | 96 | + | [18] |
| 30  | 53,686 | 2.92 | | Hexatriacontane; n-Hexatriacontane | C\(_{36}\)H\(_{74}\) | 97 | + | [18] |
| 31  | 54,598 | 0.54 | | Vitamin E; alpha-Tocopherol | C\(_{30}\)H\(_{56}\)O\(_2\) | 81 | + | [17, 22] |
| 32  | 58,976 | 1.99 | | Cholesta-8,24-dien-3-ol | C\(_{27}\)H\(_{48}\)O | 79 | + | [22] |
4. Conclusion
There were 32 bioactive compounds of *Glicidia maculata* ethanol extract which identified by GC-MS analysis. The five major components are phytol, 1-Octadecyne, 2,6,10,15,19,23-hexamethyl-Squalene, n-Eicosane and 4-Hexadecen-6-yne.

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