Journal of Associated Medical Sciences 2018; 51(1): 11-18

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Investigation of sulfated glycosaminoglycans and their agarose gel electrophoresis patterns from plant extracts

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ARTICLE INFO

Article history:
Received 20 November 2017
Accepted as revised 18 December 2017
Available online 15 January 2018

Keywords:
DMMB, uronic acids, glycosaminoglycans, agarose gel electrophoresis, spectrophotometry

ABSTRACT

Background: Many kinds of sulfated glycosaminoglycans (GAGs) play an essential role in both physiological and pathological conditions. Most of them are obtained from animal sources, and used as nutraceuticals or therapeutic applications.

Objectives: In this study, we aimed to screen for the presence of sulfated GAGs from 8 locally available plants, including ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), star fruit (*Averrhoa carambola*), Namwa banana (*Musa ABB* 'Kluai Namwa'), bitter gourd (*Momordica charantia*), purple-fruited pea eggplant (*Solanum trilobatum*), noni (*Morinda citrifolia*), and finger root (*Boesenbergia rotunda*).

Materials and methods: All plants were extracted, and sulfated GAGs from the extracts were investigated by dimethylmethylene blue (DMMB) dye-binding assay, uronic acid assay, UV-Vis spectrophotometry, and agarose gel electrophoresis.

Results: DMMB dye-binding and uronic acids assays revealed the presence of sulfated GAGs in all extracts with various degrees of sulfated GAGs levels. These results correlated to UV-Vis spectrophotometry that showed the maximum absorbance peaks from all extracts at 190-210 nm, which was similar to sulfated GAGs standard absorption. Interestingly, agarose gel electrophoresis suggested that sulfated GAGs in all extracts exhibited diverse patterns in alcian blue, toluidine blue and safranin O staining.

Conclusion: Our results indicate that all plant extracts contain sulfated GAGs at certain levels, which could be a new approach for future study in bioprospecting.

Introduction

Glycosaminoglycans (GAGs), also called heteropolysaccharides or mucopolysaccharides, are classified as carbohydrates. Their structures consist of long linear polymer of repeated disaccharide units, comprising uronic acids with either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc).[1] There are two types of GAGs depending on their sulfation: non-sufated GAGs, such as hyaluronic acid (HA); and sulfated GAGs, such as chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP), heparan sulfate (HS), and keratan sulfate (KS).[2-4] These GAGs are naturally found in animals, and most of them are incorporated with core proteins to form proteoglycans (PGs) in the extracellular matrix (ECM), or plasma membrane in various cell types. The GAGs backbone of PGs (also PGs themselves) play an important role in both physiological and pathological conditions, including inflammation, wound healing, coagulation cascade, cell behavior, tissue development, tumorigenesis and metastasis.[5-17] Owing to their functions, some medical aspects of GAGs as nutraceuticals or therapeutic applications are widely studied, and most of them are

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doi: 10.14456/jams.2018.3
E-ISSN: 2539-6056

derived mainly from animal sources.[18,19] For example, CS derived from bovine and shark cartilage is used as pharmacological agent or dietary supplement for osteoarthritis treatment.[20-23] Pharmaceutical heparin, usually obtained from bovine lung or porcine intestinal mucosa, is also used as anti-thrombotic agent.[24,25] In contrast to GAGs isolated from animal sources, little is known about GAGs isolated from plants. However, some studies have implied the presence of GAGs, and their biological effects from some plant species. For instance, there have been found GAGs which show heparin-like activity in the higher plants, including *Filipendula ulmaria*, *Paeonia anomala,* and *Paeonia suffruticosa*.[26-28] The presence of GAGs-like glycans have been observed in marine plants; *Ruppia maritima, Halodule wrightii, Halophila decipiens, Avicennia schaueriana, Rhizophora mangle*; and freshwater plants; *Eichhornia crassipes, Hydrocotyle bonariensis, Nymphaea ampla*.[29-31] Interestingly, the study of *Orthosiphon stamineus*, a medicinal plant which is widely distributed in Malaysia, and also Africa, revealed the presence of GAGs structure with uronic acids by FTIR and NMR analysis.[32] In addition, previous studied have exhibited the pressence of GAGs in crude garlic and mushroom extracts, which were detected by dimethylmethylene blue (DMMB) dye-binding assay and UV-Vis spectrophotometry.[33,34] As a consequence to these findings, we thought that screening for GAGs from terrestrial plants should be an interesting approach. Hence, the aim of this study was to screen for the presence of sulfated GAGs from 8 terrestrial plant extracts, including ginger, turmeric, star fruit, Pisang Awak banana, purple-fruited pea eggplant, bitter gourd, noni, and finger root.

Materials and methods

1. Preparation of plant extracts

One hundred grams of each sample, comprising ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), star fruit (*Averrhoa carambola*), Namwa banana (*Musa ABB* 'Kluai Namwa'), bitter gourd (*Momordica charantia*), purple-fruited pea eggplant (*Solanum trilobatum*), noni (*Morinda citrifolia*), and finger root (*Boesenbergia rotunda*) were extracted as previously described with some modifications.[35] Briefly, each sample was extracted in deionized water pH 4.5 at 37 ° C in water bath for 18 hours. Then, the extracts were boiled for 10 minutes, filtered through a muslin cloth and Whatman filter papers, and centrifuged. After that, supernatant of the extracts was collected, and the same extracts were pooled together. These crude plant extracts were then lyophilized, and stored at -20° C until they were used for the experiments. About 5 mg of each plant extract powder was dissolved in 1 mL of deionized water, and subjected to analyses unless otherwise noted.

2. Measurement of glycosaminoglycan levels

The levels of sulfated GAGs in the extracts were determined by 1,9-dimethylmethylene blue (DMMB) dye-binding (Sigma-Aldrich, USA) assay using chondroitin sulfate (CS) (Sigma-Aldrich, USA) as standard.[36] Briefly, 50 µL of either the extracts or standards were added into 96-well plates. Next, 200 µL of DMMB solution was added, and the mixtures were mixed. Then, the absorbance was read at 630 nm. The levels of GAGs were estimated by the calibration curve of chondroitin sulfate standards against their known concentrations.

3. Measurement of uronic acid levels

The levels of uronic acids in the extracts were measured by carbazole reaction using D-glucuronic acid lactone (Sigma-Aldrich, USA) as standard.[37] Briefly, 60 µL of either the extracts or standards were incubated with 300 µL of carbazole reagent A at 100° C for 15 minutes. Then, they were cooled down in ice slurry. After that, the mixtures were added with 12 µL of carbazole reagent B, incubated at 100° C for 15 minutes, and cooled down. The absorbance was read at 562 nm. The levels of uronic acids were estimated by the calibration curve of D-glucuronic acid lactone standards against their known concentrations.

4. UV-Vis spectrophotometry analysis

The extracts were examined by spectrum mode on UV-Vis spectrometer (UV2401, Shimadsu, Japan), using chondroitin sulfate as GAGs standard. Then, standard and the extracts at 50 µg/mL in deionized water were scanned from 190 to 700 nm, and analyzed.

5. Agarose gel electrophoresis

GAGs from the extracts were analyzed by agarose gel electrophoresis with some modifications.[38] Briefly, loading buffer was mixed with standards and the extracts. Then, 5 mg of GAGs chondroitin sulfate standard (Sigma, USA), or 50 mg of each extract except finger root and ginger which was used at 70 mg, were applied to a 1% agarose gel having a thickness of about 5 mm, and run for 30 minutes at 70 V in TBE buffer. Next, GAGs were fixed in the gel with 0.1% N-cetyl-N,N,N-trimethylammonium bromide (CTAB) solution for overnight at room temperature. After that, air-dried the gels for 2 hours, they were then stained with 0.5% (w/v) alcian blue (Himedia laboratories, India) in 2% acetic acid for 2.5 hours, 0.1% (w/v) toluidine blue (Sigma, USA) in acetic acid/ethanol/water (1:50:49, v/v) for 2 hours, and 0.1% safranin O (Merck, USA) for 3 hours, respectively. Then, the gels were destained, and photographed by G:BOX F3 gel documentation system (GeneSys, USA).

6. Statistical analysis

Data were presented as mean with standard deviation. Student's t-test was used to analyze the association of GAGs levels in DMMB dye-binding, and uronic acids assays. The significant difference was considered at $p<0.05$.

Results

Glycosaminoglycans screening of the plant extracts

The levels of glycosaminoglycans (GAGs) in the extracts were shown in Figure 1. All extracts contained GAGs in various degrees. The highest level was observed in tumeric, followed by star fruit, ginger, banana, noni, bitter gourd, finger root, and purple-fruited pea eggplant, respectively. In addition, when compared GAGs levels of other plant extracts to ginger, the significant difference of GAGs levels

was observed in all the extracts except banana, and noni. These results suggested that the variation of GAGs levels highly depends on the kinds of plants.

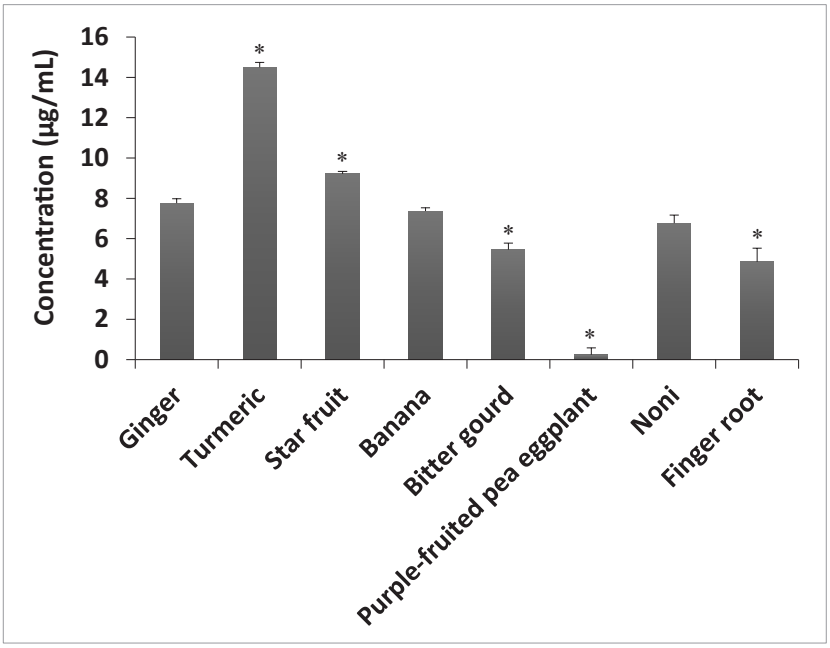

Figure 1. *Dimethylmethylene blue (DMMB) dye-binding assay of glycosaminoglycans from 8 plant extracts. Note: The levels of sulfated GAGs in the 8 plant extracts presented as mean±S.D. Asterisks (*) indicate significant difference from ginger (t- test, *p<0.05).*

Uronic acids content of the plant extracts

The levels of uronic acids in the extracts were shown in Figure 2. The highest level of uronic acids was observed in noni, followed by banana, star fruit, purple-fruited pea eggplant, bitter gourd, ginger, turmeric, and finger root, respectively. These results supported the presence of GAGs in all extracts because uronic acids were incorporated as a subunit of GAGs chains. However, when compared uronic acids levels of other plant extracts to ginger, the significant difference of uronic acids was observed only in star fruit, banana, and noni. Therefore, it seemed likely that uronic acids content was slightly different among the plants used in this study.

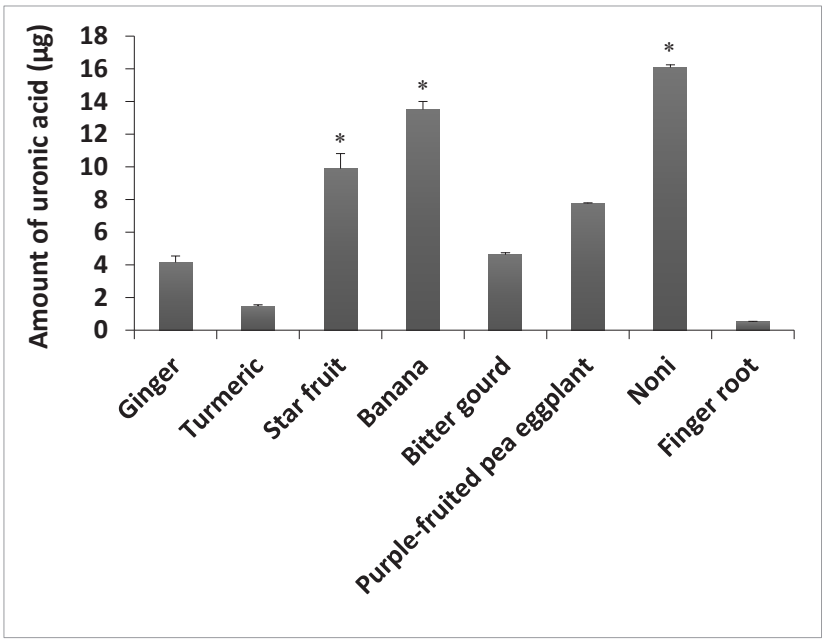

Figure 2. *Uronic acids assay of glycosaminoglycans from 8 plant extracts. Note: The levels of uronic acids in the 8 plant extracts presented as mean±S.D. Asterisks (*) indicate significant difference from ginger (t- test, *p<0.05).*

UV-Vis spectrophotometry analysis

Scanning UV-Vis spectra of the extracts was observed in Figure 3. The results demonstrated that CS standard had the maximum absorption at 190 nm (peak shown without any arrow). In addition, we also found that most extracts, including ginger, turmeric, star fruit, bitter gourd, purple-fruited pea eggplant, and noni had the same absorption patterns around 190-210 nm that resembled to CS standard, as indicated by the arrows in Figure 3, A-C and E-G, respectively. Though, the absorption patterns from banana and finger root extracts (Figure 3, D and H) showed a lesser extent of absorbance near 190-210 nm, their absorption patterns were also similar to most extracts and standard. These results suggested the presence of GAGs because the carboxylate chromophore of iduronic acid, glucuronic acid, and N-acetyl chromophores presented in the GAGs chains were found at peak 190-210 nm.[2,3,38]

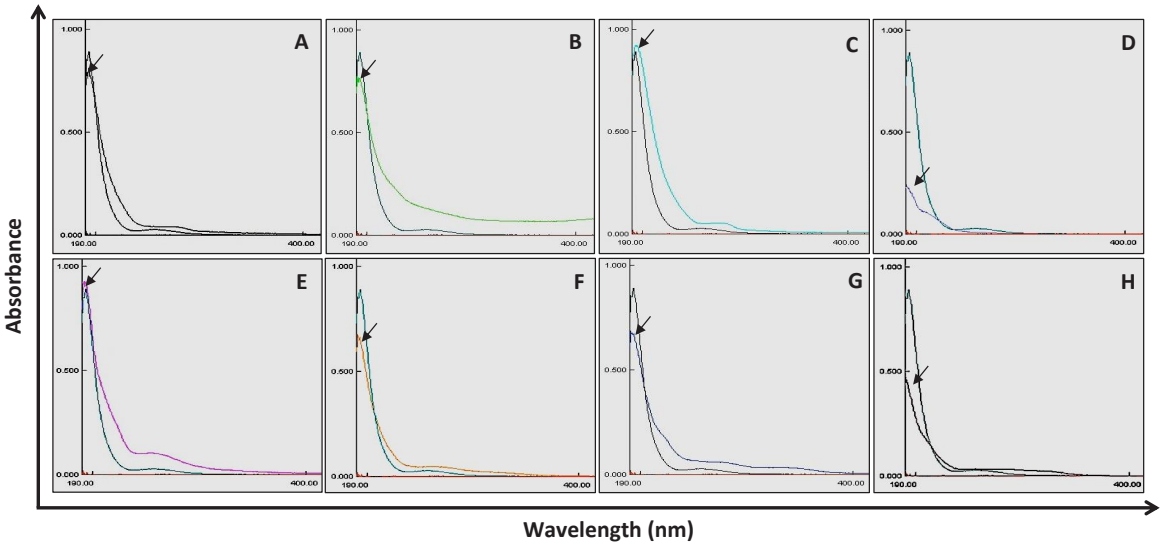

Figure 3. *UV-Vis spectrophotometry patterns of glycosaminoglycans from 8 plant extracts. Peaks of plant extracts are indicated by the arrows. A, ginger; B, turmeric; C, star fruit; D, banana; E, purple-fruited pea eggplant; F, bitter gourd; G, noni; and H, finger root.*

Agarose gel electrophoresis patterns

A qualitative analysis of the GAGs from the extracts was carried out by agarose gel electrophoresis in Figure 4. The staining pattern of CS standard (MW≈60kDa) was seen in lane 1 (Figure 4, A-C). For alcian blue staining, there was considerably faint band of GAGs from ginger extract above standard, and there were 2 bands of GAGs from turmeric extract; one band was above, and the other band was below standard (Figure 4A, lane 2 and 3). In contrast, for toluidine blue and safranin O stainining, there were bands of GAGs from ginger and turmeric extracts presenting near the site of standard (Figure 4B and 4C, lane 2 and 3). The staining patterns of the extracts, comprising star fruit, banana, purple-fruited pea eggplant, bitter gourd, and noni similarly exhibited large bands of GAGs above and overlap standard (Figure 4B and 4C, lane 4-8) except the small faint bands of GAGs from finger root extracts that appeared near the site of standard (Figure 4B and 4C, lane 9), which could be observed in all kinds of the staining dyes. These results indicated the presence of GAGs at different sizes from the extracts.

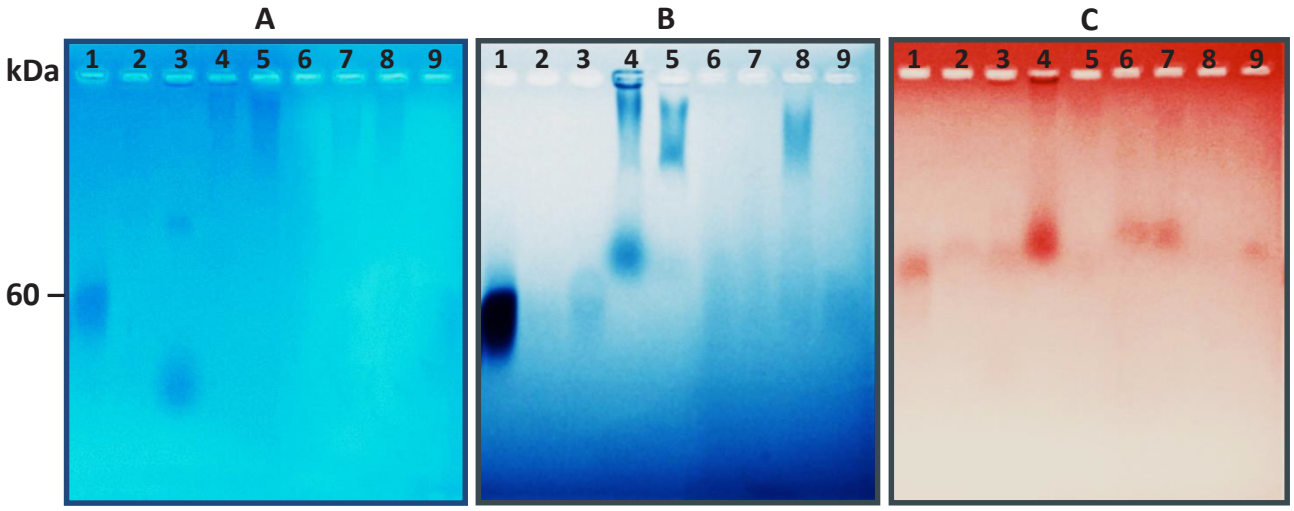

Figure 4. *Agarose gel electrophoresis of 8 plant extracts. A, alcian blue staining; B, toluidine blue staining; and C, safranin O staining. Lane 1, standard chondroitin sulfate (~60 kDa); Lane 2-9, ginger, turmeric, star fruit, banana, purple-fruited pea eggplant, bitter gourd, noni, and finger root, respectively.*

Discussion

In this study, we screened for the presence of GAGs from plant extracts by DMMB dye-binding and uronic acid assays. DMMB dye-binding assay is the most common cationic dye used to quantify sulfated GAGs in tissue samples, culture media, and bodily fluids.[36,39,40] The ability of DMMB method to detect GAGs is based on the binding to sulfated GAGs, and results in DMMB dye shift.[41] However, the limitation of this method is the interference from other polyanionic substances that can cause false negative results.[42] Since the levels of uronic acids from purple-fruited pea eggplant extract was rather high, the very low levels of GAGs from purple-fruited pea eggplant extract detected by DMMB dye-binding method could be due to some interferences. The simple subunit of GAGs is composed of long chains of repeated disaccharide units, comprising uronic acids with either GlcNAc or GalNAc.[1] Thus, the measurement of uronic acids is another method that can be used for GAGs screening.[37] However, there have been reported that detection of GAGs by the measurement of uronic acids through carbazole reaction is less sensitive than DMMB dye-binding method, and may be interfered by some sugars or salts.[42-44] These may lead to the explanation why the levels of uronic acids in each extract are not relatively shown as the similar trend levels of GAGs measured by DMMB dye-binding assay. For spectrophotometric analysis, it is thought to be the consequence of the carboxylate chromophore of iduronic acid, glucuronic acid, or N-acetyl chromophores at peaks 190-210 nm which suggested the presence of GAGs in all extracts. This evidence supports the presence of GAGs detected by DMMB dye-binding, and uronic acids assays. Nevertheless, previous studies found that the peaks of these chromophores are only slightly shifted among different GAGs types.[45, 46] Hence, it seems unlikely that this method could specifically distinguish the types of GAGs.[2, 3, 38] Though each method, including DMMB dye-binding method, uronic acids assay, and spectrophotometry has some limitations, a detection of GAGs is still reliable when thoroughly interpreted the correlation among these methods. Thus, further investigation of GAGs should be considered to implement other reliable analytical techniques, such as high-performance liquid chromatography (HPLC), magnetic resonance spectroscopy (NMR), mass spectrometry (MS), and disaccharide analyses.[40]

Agarose gel electrophoresis is one of the most qualitative and quantitative techniques used to evaluate GAGs in the mixtures.[47] It is also applied for various purposes, such as separation of GAGs from tissues, organs, or biological fluids, determination of GAGS content and purity for pharmaceutical application, utilization in the medical field for screening monitoring, and diagnosis for mucopolysaccharidoses (MPS).[40, 47-49] In this study, the presence of sulfated GAGS were confirmed by an agarose gel electrophoresis. After their separation, the visualization of sulfated GAGs are based on their negative charged carboxyl and sulfate groups binding to cationic dyes, such as alcian blue, toluidine blue, and safranin O. Our results showed that the staining patterns of most extracts, including star fruit, banana, purple-fruited pea eggplant, bitter gourd, noni, and finger root were similarly observed in all 3 staining dyes. In contrast, alcian blue staining patterns of sulfated GAGs from ginger and turmeric extracts were different from the patterns seen in the other dyes. These are perhaps cause by some substances in the extracts, which are still intriguing for further study. Though, all 3 cationic dyes can stain sulfated GAGs and show the similar patterns of staining GAGs, we observed various degrees of more intense staining of GAGs from toluidine blue, alcian blue, and safranin O, respectively. This is probably due to the fact that toluidine blue has a higher affinity for the sulfur compared to safranin O.[50] Also, toluidine blue and alcian blue have different limit of detection for sulfated GAGs staining because it depends on the procedures used.[47] Thus, staining by toluidine blue is particularly suited for our study. As we did not have standards of several GAGs types with various molecular mass, we used CS-C standard for the estimation of GAGs size. It seems likely that the size of GAGs found in most extracts were supposed to be ≥60 kDa because the results exhibited large bands of GAGs above and overlap the area of standard. However, it could not surely estimated the size of GAGs, for these crude extracts are not well treated or purified. To specifically identify the size of actual GAGs present by electrophoresis separation, it is needed to purify the extracts, treat with GAGS degrading enzymes, i.e. chondroitinase ABC, heparin lyase, or heparinase, and it is then subjected to electrophoresis with different molecular mass of standards.

GAGs are mainly found in animals, particularly in vertebrates. They are widely studied in both physiological and pathological circumstances, and also used as nutraceuticals or therapeutic applications in some certain conditions or diseases.[5-19] Due to these extensively studies, they are well known for their structures which consist of repeating disaccharides units of uronic acids with either GlcNAc or NGalNAc with distinct sulfation pattern.[1-4] In contrast, little is known about the structure of GAGs and their biological functions from plants; however, some studied have reveled the presence of GAGs or GAGs-like glycans from some plant species. For example, the presence of GAGs-like glycans have been observed in marine plants.[9, 10] In addition, monosaccharides found in GAGs-like glycans from the root of freshwater plants, especially *Eichhornia crassipes*, contain galactose, glucose and arabinose.[29] A terrestrial medicinal plant, *Orthosiphon stamineus*, also contains uronic acids as a subunit of GAGs structure.[32] Besides these plants, it is interesting that there is another rich non-animal source of sulfated glycans obtained from seaweeds. In seaweeds, the structure of sulfated glycans depends on the type of them. For instance, the most well known brown seaweed contains sulfated fucans that mostly composed of α-L-fucose polymers.[51] Green and red seaweeds contain sulfated galactans which mosly contain β-D-galactose polymers.[51] It is noticed that most sulfated glycans found in brown, green, and red seaweeds are composed of repeating disaccharides units with several patterns of sulfation. They also exhibit various biomedical functions in inflammation, coagulation, angiogenesis, cell adhesion, and involve many pathophysiological systems.[51] Taken together, the structures of GAGs or sulfated glycans from plants, and another non-animal source, such as seaweed

usually contain long chains of simple sugars polymers, which rather different from GAGs polymers of animals. However, they are all similar in term of sulfation. In this study, we measured GAGs by DMMB dye-binding method, using animal GAGs as standard. As mentioned above, the structures of GAGs from plants are rather different from animals GAGs polymers, but all of them still have degree of sulfation. Since the ability of dye-binding method to detect GAGs is based on the binding to sulfated GAGs, we think that any sulfated glycans from non-animal sources could be quantified by this method. In addition, de Araújo et al. also performed DMMB dye-binding method to monitored sulfated polysaccharides obtained from red seaweed.[52] On the other hand, we also realize that it would be more reliable if we use non-animal standard to estimate the concentration of GAGs from plants for future study.

As we could observe from our current study, we think that the tentative potential benefits of GAGs obtained from the plant extracts may be further used as a source of non-animal nutraceuticals, especially they may be useful for vegetarian diet. However, we could not surely conclude whether it can be used instead of animal GAGs because some studies about the purity, structure, and clinical trials of them are still left to be elucidated.

Conclusion

Our results have shed new light on the investigation of sulfated GAGs that contain in the plant extracts. This evidence supports the view that plants heteropolysaccharides as bioprospecting should not be disregarded.

Conflict of interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgements

We would like to express our sincere thanks to division of microbiology for the gel documentation system support. We also would like to thank Dr. Preeyanat Vongchan, division of transfusion science, for standard glycosaminoglycans supply, and Thailand excellence center for tissue engineering and stem cells, department of biochemistry, faculty of medicine, for technical assistance and consultation.

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