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Research Article

A RAPID QUANTITATIVE DYE-BINDING METHOD OF SCREENING GLYCOSAMINOGLYCANS PRESENCE IN MEDICINAL PLANTS

CHE NUR MAZADILLINA ZAHARI, MARINA MOHD SHAM, SAKINA SHAHABUDIN, MOHD-HAIRUL AB RAHIM AND NINA SUHAITY AZMI*

Department of Industrial Biotechnology, Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, 26300 Kuantan, Pahang, Malaysia. Email: nina@ump.edu.my

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ABSTRACT

Objectives: The aims of this paper are to extract glycosaminoglycan (GAG) from four local medicinal plants and to characterize the crude extract with highest total sulfated GAG to reduce the dependency of using animals as major sources.

Methods: Crude GAG was extracted from four plants (*Gaultheria procumbens, Strobilanthes crispus, Orthosiphon stamineus,* and *Ficus deltoidea*) using hot water extraction with some modifications. Ultraviolet (UV) spectrophotometry was conducted for purity test. Total sulfated GAG was determined using Blyscan assay kit. By comparing results between the extract yields and total sulfated GAG, the plant consisting of high total sulfated GAG was chosen for further characterization. The selected plant sample was examined by microscopy and further analyzed by nuclear magnetic resonance (NMR) and Fourier-transform infrared (FTIR) spectroscopy.

Results: All four plants showed absorbance peaks between 214 and 232 nm in UV scan that represented negatively charged sugar. *O. stamineus* was found to contain the highest amount of sulfated GAG, 62.63±0.01 µg/mg by Blyscan assay. Microscopical examination confirmed the identity of *O. stamineus* sample by comparing to the reference. Both NMR and FTIR analysis of *O. stamineus* crude yield showed the presence of hydroxyl, sulfates, carboxylate, and amine groups, suggesting close resemblances to GAG structure.

Conclusion: The results suggested that all four plants contained GAG compound. *O. stamineus* was found to exhibit the most abundant total sulfated GAG and has the potential to become a new plant-based source for GAG.

Keywords: Blyscan assay, Glycosaminoglycan, Medicinal plants, *Orthosiphon stamineus,* Plant-based source.

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INTRODUCTION

Glycosaminoglycan (GAG) is a type of sugar that widely applied in diverse fields such as cosmetics, supplementary diet, food, and textiles. GAG has five different classes (chondroitin sulfate, heparin, heparan sulfate, dermatan sulfate, and keratan sulfate) and each of them carries specific function that can be recognized from sulfated group presence, except for a unique class known as hyaluronic acid [1-5]. They are negatively charged components belong to polysaccharide group (mucopolysaccharide) and made up of repeating disaccharide units. Consisting of amino sugars, N-acetyl glucosamine, and N-acetyl galactosamine as the repeating units, they are built up along with uronic acid (iduronic acid and glucuronic acid) [6]. In the medicinal area, GAG helps to prevent blood coagulation through biological action carried by heparin that inhibits coagulation process, especially in hemodialysis. GAG is one of the components used to construct dressing to support wound healing [7].

Since GAG is comprehensively utilized worldwide, GAG supply is in constant demand. Porcine is one of the major sources of GAG, but there is a shortage of porcine supply due to its extensive application in other areas as well. Compounds from other animal sources, including marine lives, tend to be easily affected by infectious viruses [3,4]. For example, there is a risk of contracting mad cow disease using cow as a source of GAG [8]. Therefore, using plant source may offer less risk of infections in human.

Since ancient times, local medicinal plants in Malaysia have offered many functional benefits to humans such as wound healing, reducing fever, curing hypertension and diabetes, and treating inflammations. Medicinal plants have become a fundamental source not only in Malaysia but are also utilized by almost 3.4 billion of humankind around the world who depend on natural-based products [9]. Reasonably, medicinal plants can be considered as both safe and effective toward achieving an optimum cure without long-term side effects like those usually resulted by modern drugs [10,11]. Considering the awareness of the communities on the importance of these natural treasures, researchers have come out with many studies regarding the functions of natural compounds. Numerous studies have been done on animal extracts since decades, yet there are limited reports on GAG presence in local medicinal plants [12]. In this study, four types of medicinal plants that are known for their benefits in traditional herbalism were chosen and screened for their potential of containing GAG.

Here, the GAG content in plant extracts was quantified using Blyscan assay, a dye-binding method that allows for rapid and accurate quantitative as well as a qualitative measurement for GAG. This assay can determine the total content of sulfated GAG in samples, but not each specific GAG class. On contact with dissociation reagent from Blyscan kit, samples would show calorimetric reaction by giving off darker color, which indirectly represented GAG presence in the samples. Use of Blyscan assay is more simple and convenient to screen for GAG presence in the extracts from medicinal plants. The GAG extract from the selected plant with the most GAG content was characterized using Fourier-transform infrared (FTIR) and nuclear magnetic resonance (NMR). This current study is striving to find a novel plant-based source for GAG that can replace porcine or other animals as sources of GAG.

METHODS

Plant material preparation

Four local medicinal plant samples (*Gaultheria procumbens, Strobilanthes crispus, Orthosiphon stamineus,* and *Ficus deltoidea*) were supplied by Delima Jelita Herbs Enterprise situated in Kedah, Malaysia. The plant samples were acquired in the form of the whole plants and came in powdered form.

Hot water extraction

The extraction method was adapted and modified from two previous studies [13,14]. 20 g of each sample was dissolved in 400 ml of distilled water. The solutions were mixed at 80–95°C for approximately 15–30 min, depending on the texture of the samples. After cooling down, the plant samples were centrifuged at 10,000 rpm for 10 min, and followed by filtration. The residue was discarded while filtrate was collected and kept at −80°C before lyophilization. After freeze-drying, the samples (in powdered form) were concentrated using 70% ethanol (v/v). The GAG yield was maintained at a cool and dry place, stored at 4°C, and its raw powder was kept at room temperature.

Ultraviolet (UV) screening

About 2 mg of each lyophilized plant sample was diluted in 2 ml of distilled water as a stock solution. Then, about 2 µl of the plant's stock solution was diluted into 2 ml of distilled water. During the preparation of the stock solution, the sample was vortexed for a few seconds to make sure the sample blended well before analysis. Solution of 99.9% methanol was used as a blank and pipetted into a glass cuvette. The cuvette was wiped for a clearer surface to avoid reading interference. The sample aliquots were pipetted into a cuvette and observed under UV spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). The absorbance was read between 200 and 800 nm and obtained in triplicates.

Blyscan assay

Blyscan test was conducted using 96-well microplate, with distilled water as a blank. Each 50 µl of plant sample was transferred into microcentrifuge tubes and topped up until 100 µl. Then, 1 ml of Blyscan dye reagent was added into each centrifuge tube and vortexed for 30 min. As an observation, the samples with sulfated GAG may turn into bright pink whereas no change was observed for the absence of GAG. After the samples were centrifuged at 12,000 rpm for 10 min, the supernatant was removed. Approximately 0.5 ml of dissociation agent was added to the samples and vortexed until they mixed thoroughly. To remove foam that formed due to the vigorous vortex, samples were centrifuged for 5 min. An aliquot of 200 µl was pipetted into each well of the microplate. The absorbance was read on a microplate reader (Tecan Infinite M200 Pro, Zürich, Switzerland) at 656 nm. A calibration curve was plotted with chondroitin sulfate as a standard.

Microscopy examination

O. stamineus plant was purchased from Taman Pertanian Jubli Perak Sultan Haji Ahmad Shah, Bandar Indera Mahkota, Kuantan, Malaysia and is maintained at the greenhouse of Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM) Kuantan. The aerial parts were collected, dried in an oven at 50°C, powdered and was used as a reference in the microscopical examination of the powdered sample. The various cell structures were determined using methylene blue for general observation, and the starch granules were identified using Lugol's iodine solution. The images were digitally captured with a Leica DM750 microscope (LeicaTM-Wetzlar, Germany) using a video camera plugged to a computer utilizing the Leica Application Suite EZ (LeicaTM-Wetzlar, Germany) software for image analysis. All microscopical pictures were taken with ×400 magnification. The microscopical characters of the powdered form of *O. stamineus* sample were examined under microscopy and compared to the *O. stamineus* reference.

FTIR

Crude samples were analyzed using FTIR spectrophotometer (Perkin Elmer Spectrum 100, MA, USA). A fraction was taken and ground with potassium bromide powder. To obtain a transparent result for viewing, the fraction must be pressed into pellets before undergoing measurement within the frequency range of 4000–400 cm^{-1}.

NMR

Approximately 100 mg of crude GAG was dissolved in 0.5 ml of 99.8% deuterium oxide after microfiltration through 0.45 µm nylon filters. Sonification for 60 s was done to remove air bubbles from the solution. Crude samples were sent to Central Laboratory, Universiti Malaysia Pahang for NMR analysis (Bruker Ultra Shield Plus 500, MA, USA) using supersaturation method and Tetramethylsilane was used as internal reference.

Statistical analysis

The plant samples were analyzed in triplicates, and the results were reported in mean ± standard deviation values.

RESULTS

In this study, GAG was extracted from four medicinal plants by hot water extraction. The initial weight of the whole plant used for the plant extraction was 20 g. Crude GAG was successfully extracted and recorded in Table 1.

Purity test of crude extracts was done using UV scan to identify the presence of other compounds than sugar in tested plants. As this study did not proceed to purification step, macromolecule such as protein was expected to be present. Somehow, the macromolecules may not affect the readings of an assay conducted. The UV scan profile (Fig. 1) shows strong absorbance peaks between 200 and 250 nm and some plants also showed a broad signal around 270–300 nm wavelength. Absorbance peak could be seen in all plants between 214 and 232 nm that represented negatively charged sugar. One distinct group can be easily identified as carboxylate chromophore between 220 and 230 nm [15]. A feature in UV scan spectra (around 280 nm) of the plants was exhibited by protein presumably linked to the GAG structure. The rest of spectrum was considered as fingerprints.

Total sulfated GAG was determined in Blyscan assay using absorbance values expressed through a calibration curve constructed from chondroitin sulfate standard. Based on the results, all four plants possessed promising GAG content and predominantly proven the ability of the listed plants as GAG sources. Total sulfated GAG content that was reckoned from whole crude GAG for each respective plant extract was shown in Table 2. Out of four tested plants, *O. stamineus* produced the highest total sulfated GAG from the crude yield.

Since *O. stamineus* emerged with the highest amount of total sulfated GAG, further characterization was performed only on *O. stamineus*. Based on visual observation, the general morphology of the *O. stamineus* plant

Table 1: Crude yield of GAG extracts from four medicinal plants

Plant samples	GAG yield of extracts (g)
Ficus deltoidea	1.06±0.02
Gaultheria procumbens	4.17±0.00
Orthosiphon stamineus	1.47±0.00
Strobilanthes crispus	0.67±0.00

Data are mean±SD values; n=3. GAG: Glycosaminoglycan, SD: Standard deviation

Table 2: The total sulfated GAG (µg/mg) calculated from their respective crude yield

Plant samples	Total sulfated GAG in crude yield (µg/mg)
Ficus deltoidea	40.61±0.00
Gaultheria procumbens	20.71±0.00
Orthosiphon stamineus	62.63±0.01
Strobilanthes crispus	9.68±0.01

Data are mean±SD values; n=3. GAG: Glycosaminoglycan, SD: Standard deviation

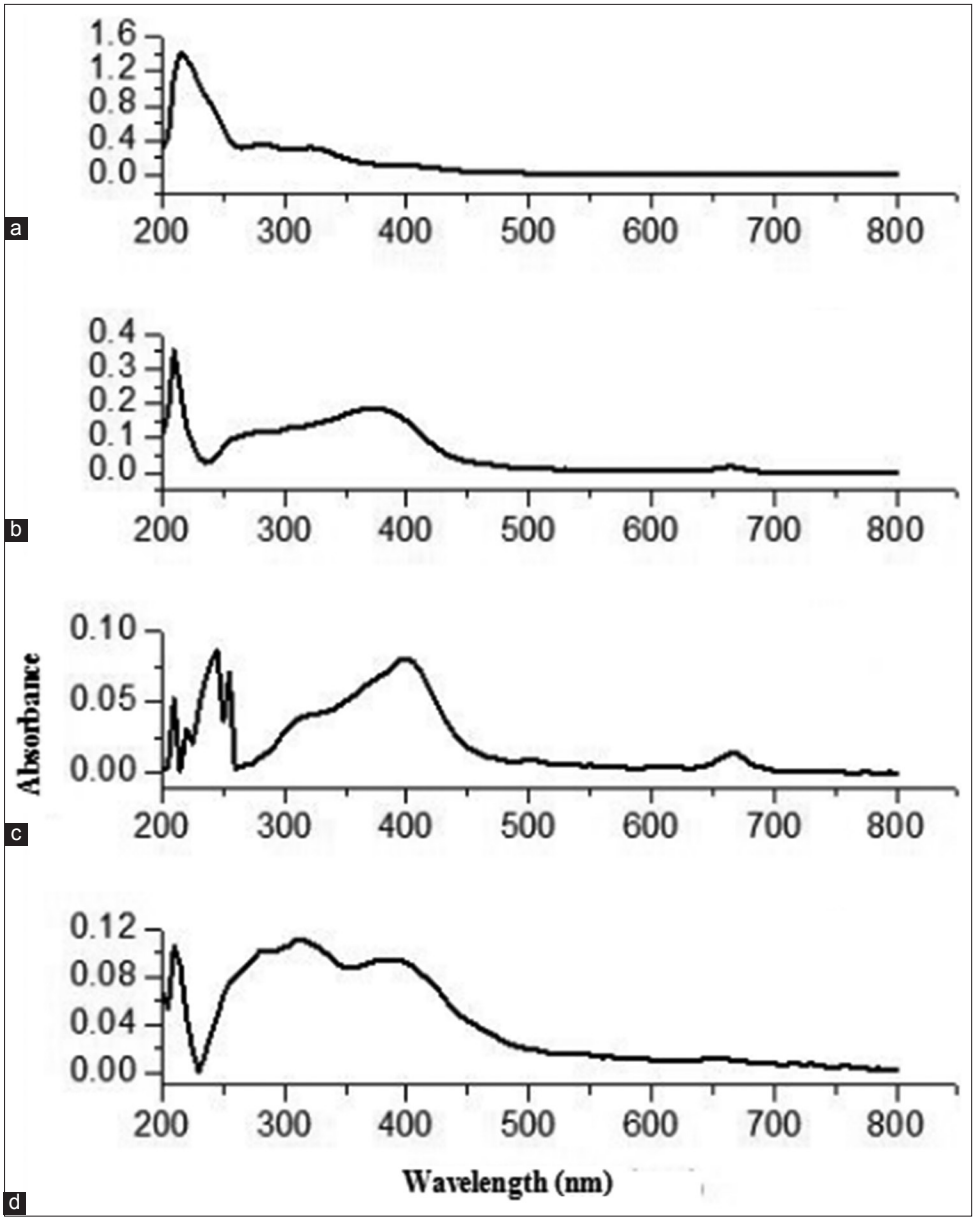

Fig. 1: Ultraviolet spectra of crude glycosaminoglycan extract from (a) *Ficus deltoidea*, (b) *Orthosiphon stamineus,* (c) *Gaultheria procumbens,* and (d) *Strobilanthes crispus*

was listed. The plant grows multi-branched, with quadrangular stem, and ascending to erect. The leaves are simple, green, with glabrous and a serrate margin, rhomboid shape, and acuminate apex and obtuse base with light green reticulate-pinnate venation (Fig. 2). The bracts, which have short length (1–2 mm) with green color, normally hold a cluster of six flowers growing in whorls along the floral axis. The flowers have bell shape, white corolla with long wispy stamens, with an unequal length that measures from 4.7 cm to 5.2 cm. This appearance makes the flowers seem to be like cat's whiskers and can grow up to 6.2 mm in length (Fig. 2). It has irregular gamosepalous calyx and corolla lobes, which covered with minute white hairs. Each flower has four stamens that are inserted near the base of the corolla tube. The fruit pod contains four oval-shaped seeds with hard external coat and rough surface [16].

For the authentication of *O. stamineus*, the microscopical characters of the powdered form of *O. stamineus* sample were examined and compared to the *O. stamineus* reference (Fig. 3). The same microscopical characters that include sinuous epidermal cells, diacytic stomata, parenchymal cells, uniseriate trichomes, tracheids, starch granules, and fragments of vessels with spiral and reticulate thickening were observed in both powdered sample and *O. stamineus* reference. Therefore, the powdered sample was authenticated as *O. stamineus*.

The *O. stamineus* crude extract was further characterized by FTIR. Characteristics peaks of *O. stamineus* at a wavelength of 4000–400 cm^{-1} are presented (Fig. 4). Crude GAG present in *O. stamineus* showed bands between 1540 cm^{-1} and 1500 cm^{-1}, suggesting protein residual [13]. The spectrum in the 3413–3250 cm^{-1} region was originated from hydroxyl group (O-H), such as those that can be found in glucuronic acid [17]. Stretching vibrations of S=O for sulfates ($^{-}SO_3$) were detected at several spectra in the range of 1312–1260 cm^{-1} from the crude sample, similar to the standard that is identical to GAG's characteristics [18]. A single peak was recorded between 1739 and 1700 cm^{-1}, suggesting carboxylate (-COO^{-})bond [19]. *O. stamineus* crude yield also showed clear peaks in the 1300–1000 cm^{-1} region, indicating amine (C-N) bond. Meanwhile, the spectrum between 2928 and 2800 cm^{-1} indicates the stretching of carbon-hydrogen bond that creates the skeleton of GAG compound.

Fig. 2: General morphology of the leaves and inflorescence of *Orthosiphon stamineus* plant (a) the leaves and (b) the inflorescence

The GAG in *O. stamineus* crude extract was also characterized in NMR. The ^{1}H NMR analysis confirmed H-4, H-5 of glucuronic acid (Glcp-UA) residue between 3.20 and 3.61 ppm (Fig. 5). This region is also comprised H-6 of N-acetyl galactosamine (Galp-Nac) residue. The signal at 1.91–1.96 ppm region suggested N-acetyl methyl of Galp-NAc residue while signal at 4.29–4.78 ppm verified the presence of H-4 and glucuronic group [20]. There was no unusual peak detected at 3.50 ppm that may indicate contaminant within sample [20]. Based on the existing peaks, some spectra showed signals for hyaluronic acid that ranges from 2.64 to 4.78 ppm which resembles protons on sugar ring. The spectra also showed the presence of heparin through 2.14–2.16 ppm signals. It can be speculated that the sample has a close resemblance to GAG compound.

DISCUSSION

This study chose to employ hot water extraction method because hot water can break plants' cell wall to bring out GAG effectively. A study on polysaccharide extraction out of Chinese herbs also applied hot water as their main solvent [21]. Other studies also reviewed that hot water extraction is significantly employed in the pharmaceutical sector, which

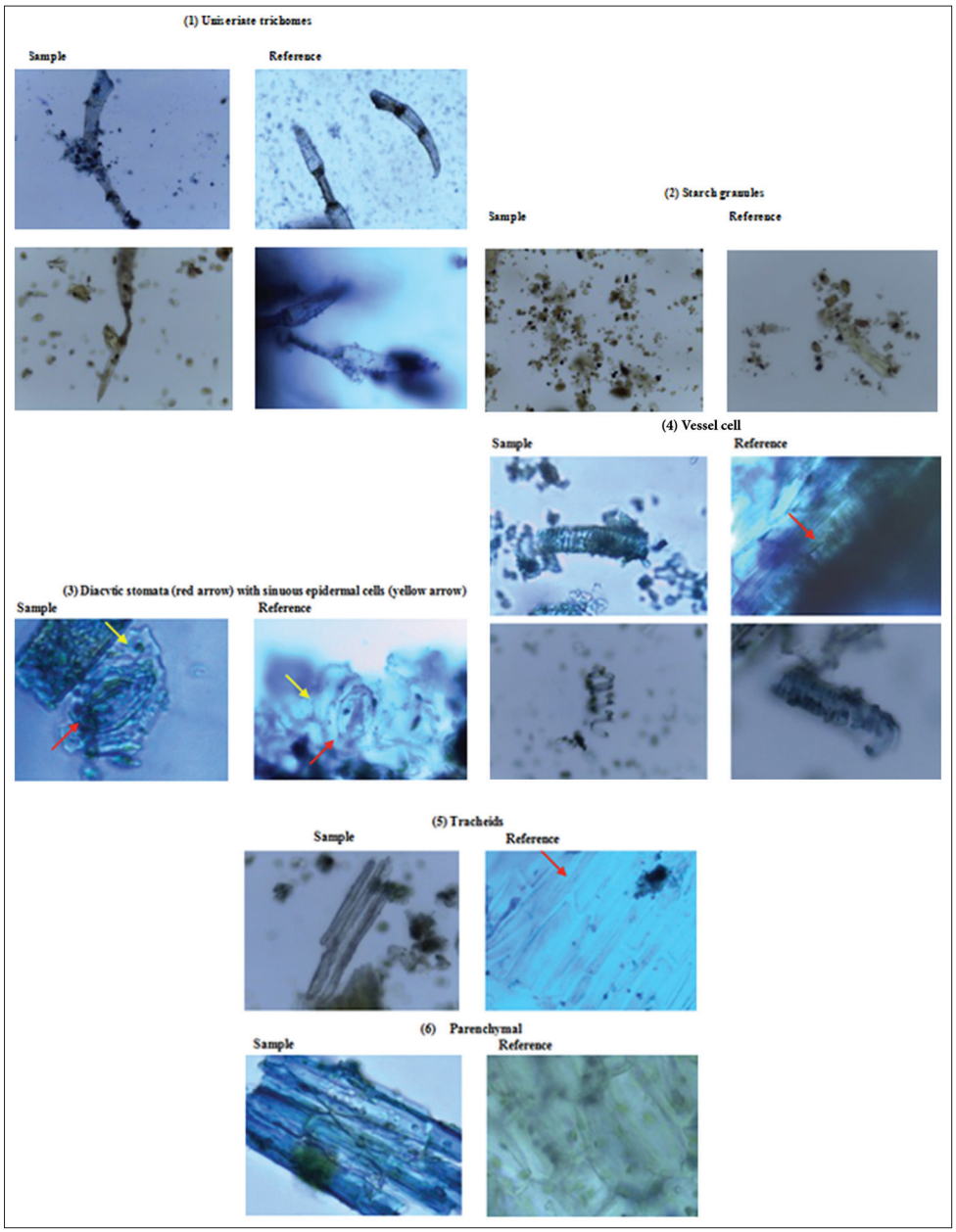

Fig. 3: Comparison of microscopical characters of *Orthosiphon stamineus* powdered form and the reference sample

meets this current study's scope [10,22]. Hot water extraction would be a better choice in industrial applications for human consumption. One study compared ethanol extraction and hot water extraction, in which the respective extract yields were further tested on mice for its survival effect on influenza A/PR/8/34 [23]. Hot water extracts showed a remarkable effect on mice survival and also on its defense ability compared to ethanol extract [23]. In addition, hot water extraction was used to elicit anticoagulant effect (one of the medical properties caused by GAG compound) in selected medicinal plants in South Africa [24]. Hot water is the most familiar extraction method especially in dealing with a bioactive compound study [25].

There are numerous ways to quantify total sulfated GAG in samples, including spectrophotometric assays. Most of these assays work by detecting sulfation level based on the changes of absorption spectrum on specific dyes [26]. For example, 1,9-dimethylmethylene blue (DMMB) assay that is shown to be effective for both animal and plant samples [12,27]. Alas, this assay has drawbacks in term of its stability and easily affected by some interferences such as protein residue [26]. Other spectrophotometric assays may use Alcian blue dye that works in the same principle as DMMB but involves more tedious and time-consuming protocol [28]. Thus, the current study chose to employ Blyscan assay with simpler and more conventional procedures. Blyscan assay has been used for all types of sample, for instance, quantification of GAG amount in nanofibrous and cell pellet [29]. Another study determined chondroitin sulfate out of synovial fluid by Blyscan assay [30]. In addition, a cancer study on mice discussed about sulfated GAG present in mice could be detected by specific binding of cationic dye in Blyscan assay toward sample [31]. These previous studies justified the application of Blyscan assay for GAG determination in the current study.

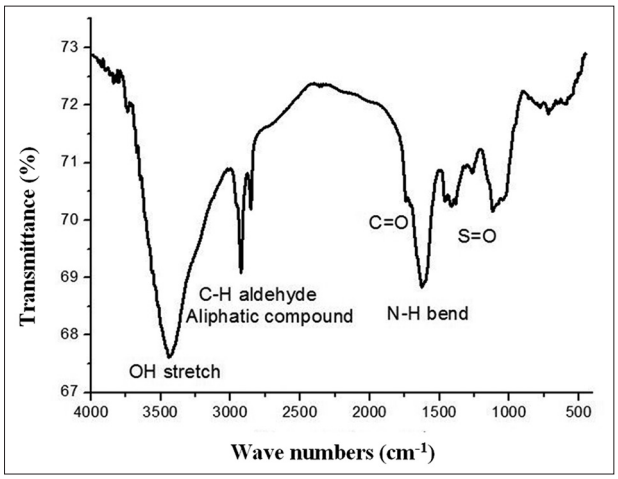

Fig. 4: Fourier-transform infrared spectra of crude glycosaminoglycan extracted from *Orthosiphon stamineus*

Here, *O. stamineus* was shown to contain the highest total sulfated GAG as compared to other three plants. From the previous study that investigated extraction and quantification of GAG from *Acanthaster planci* starfish, the highest GAG amount was found in starfish's body coelomic fluid at 55.79±0.65 µg/mg [32]. By comparison with *A. planci*, *O. stamineus* has potential to give much higher GAG amount. Another research that studied about one of the GAG classes, heparin, from seaweed showed that seaweed contained 2.3 mg/50 g of sulfated GAG, in which seaweed exhibited lower value when compared with *S. crispus* in this current study [33]. Overall, these two sources of GAG (seaweed and starfish) showed relatively smaller values of GAG than *O. stamineus.* Even though *G. procumbens* yielded about 3 times higher crude GAG extracts than *O. stamineus*, somehow, the latter plant exhibited much higher total sulfated GAG from its crude yield than *G. procumbens*. The reason for this could be possibly due to the chemical constituents that are present in the respective medicinal plants.

Cell proliferation is one of the abilities possessed by all four medicinal plants tested in the current study. It can be seen though the properties such as anticancer, anticoagulant, antioxidant, anti-inflammatory, and wound healing. *G. procumbens,* also known as Sambung Nyawa in Malay language (meaning of "prolongation of life"), is commonly consumed raw or used in cooking for its beneficial medicinal properties. This plant has been shown to have cytotoxic activity on cancer cells [34]. *F. deltoidea,* or Tabat Barito, has been traditionally made as a treatment for diabetes mellitus in regulating blood sugars. Studies were done on *F. deltoidea* for the presence of antidiabetic, antioxidant, and antihyperglycemic properties [35,36]. *F. deltoidea* also contains chlorin-type alkaloid compound that can inhibit breast tumor MCF-7 cells [37]. *S. crispus,* or Pecah Beling, shows antidiabetic properties when tested in hyperglycemic rats [38].

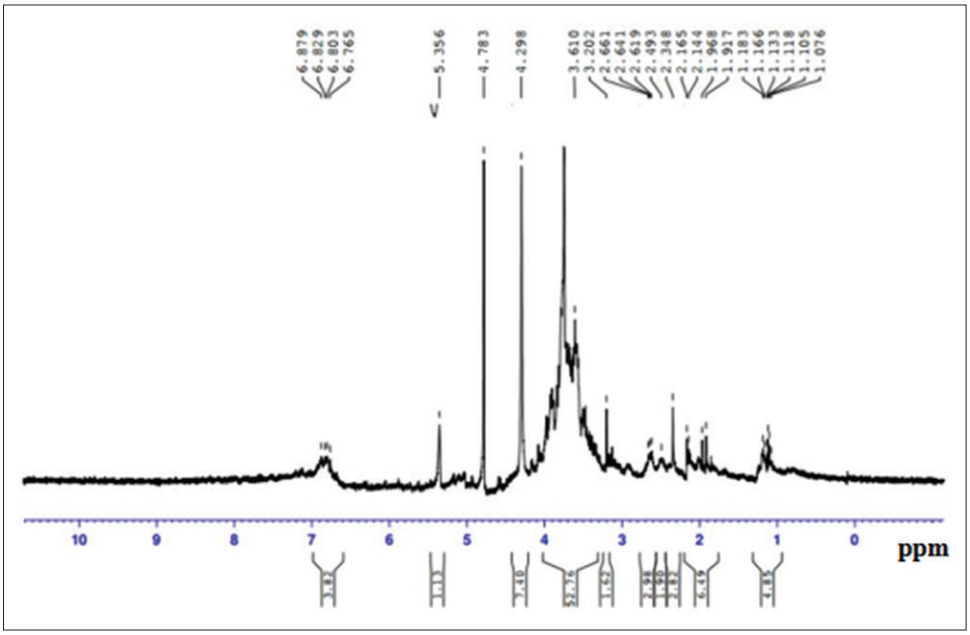

Fig. 5: Nuclear magnetic resonance spectra that correspond to disaccharide sequences of crude glycosaminoglycan extracted from *Orthosiphon stamineus*

Meanwhile, *O. stamineus,* or Cat's Whiskers, could trigger diuretic and uricosuric actions when treated in rats [39]. Leaf extracts of *O. stamineus* displayed weak inhibition of biofilm formation of *Staphylococcus aureus* [40]. One of the main constituents in the *O. stamineus* plant leaf extracts, namely polyphenols, contributes to its antioxidant potential [41,42]. *O. stamineus* also possesses active components such as terpenoids [43]. The polyphenols, which are mainly based on the plant's leaves, contribute the most in the active biological activity in *O. stamineus* [44]. Thus, the secondary metabolites compounds in *O. stamineus* such as terpenes are responsible to induce the plant's capability as anti-inflammatory and antioxidant, as well as promote cell proliferation [45]. Based on a phytochemical study, *O. stamineus* contains many other compounds such as saponins, organic acids, chromeno, and myo-inositol [46]. Further phytochemical studies on these medicinal plants may reveal more of their therapeutic benefits.

In the characterization of the plant samples, UV scanning is necessary for rough identification of the compounds that exist in the samples. UV scanning can be conducted to determine sample purity [15]. This spectroscopy can also help to distinguish GAG compounds from other sugar types. This current study applied water extraction technique, so the same solvent was used as the blank and for sample dilution. Other than ethanol, hexane or cyclohexane, it is important to avoid using other solvents with double or triple bonds that may interfere absorbance readings due to their molar concentrations. Although noises were expected to appear in the UV spectra since the crude GAG extracts were not purified in this study, their presence did not interfere the analysis.

Characterization by FTIR analysis confirmed the identity of *O. stamineus* crude extract as an aliphatic compound due to the presence of spectrums below 3000 cm^{-1} wavenumbers. FTIR can be used to identify the organic functional groups contained in the crude extract, allowing for rapid and reliable detection in diverse sample types [17,47]. The *O. stamineus* crude extract showed several absorbance peaks that indicate amine, carboxyl, hydroxyl, carbonyl, and sulfate groups. Even though some protein residual was detected, they did not cause interference in assays conducted. The presence of protein residual could be attributed to the fact that the *O. stamineus* extract had not been purified and was still in crude form. Subsequently, NMR was conducted to determine the skeleton of compounds found in the *O. stamineus* plant. NMR is a suitable instrument in providing chemical composition as well as the structure of biological systems [13]. The NMR analysis showed the ranges of peaks representing functional groups that are present in a GAG structure, suggesting that crude compound extracted from *O. stamineus* are potentially GAG. Close similarity of the signals exhibited during characterizations using FTIR and NMR could be seen. These results supported the evidence that the crude extracts from *O. stamineus* were indeed GAG. Thus, this study chose *O. stamineus* as the best plant for its promising amount of sulfated GAG.

CONCLUSION

The GAG was isolated from four types of local medicinal plants. Within this study, polysaccharides were extracted using hot water extraction while the amount of GAG was reckoned from the mechanism of dye present in Blyscan kit and analyzed through a calibration curve. *O. stamineus* plant produced the highest amount of sulfated GAG from an aliquot taken compared to other three medicinal plants. Based on FTIR and NMR analysis, the *O. stamineus* crude yield showed resemblances to GAG structure with absorbance at regions indicating the presence of hydroxyl, sulfates, carboxylate, and amine groups. The tremendous amounts of GAG that could be extracted from medicinal plants showed huge potential of becoming a successful source of GAG and subsequently can replace animal sources.

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AUTHORS' CONTRIBUTION

CNMZ performed extraction, GAG quantification, characterization by NMR and FTIR, data interpretation, and wrote the manuscript. SS contributed for interpretation of the data and manuscript preparation. MSM and MHAR contributed for plant extraction. NSA supervised the laboratory work, initiated for research design, and proofread the manuscript.

CONFLICTS OF INTEREST

The authors hereby declare that no conflicts of interest are involved that might influence the results and/or discussion reported in this manuscript.

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