

PROCESS FOR EXTRACTING *HEVEA* LATEX POLYSACCHARIDES FROM
EFFLUENT OF THE RUBBER MANUFACTURING INDUSTRY,
POLYSACCHARIDE EXTRACT, COMPOSITION AND USE THEREOF

Technical Field

5 The present invention relates generally to the field of polysaccharides extraction from *Hevea* latex. More particularly, the invention relates to the extraction of *Hevea* latex polysaccharides (HLPs) from the effluent (wastewater from the rubber sheeting process) of the Para (natural) rubber manufacturing industry.

Background Art

10 Thailand is world No. 1 natural rubber exporter. In 2018, 32,000 square kilometers (about 7.9 million acres) of rubber plantation produced over 4.8 metric tons of rubber. The focus of the rubber industry in Thailand has always been rubber as a raw material to be exported to consumer product manufacturers in other industries.

 In the industrial process of converting rubber coagulum into sheet or block
15 rubber, effluent is generated. Natural Rubber Latex (NRL) comprises about 65 to 70 per cent water (main component of the Natural Rubber Serum, NRS) and 30 to 35 per cent rubber to begin with. The NRL is usually diluted with water before being coagulated with formic acid and then calendered in order to obtain sheet or block rubber with suitable texture for further manufacturing. This effluent, being endowed with biomolecules such
20 as sugars, proteins, and lipids, plus a few minerals, presents an ideal condition for microbial growth. Water treatment is therefore required in order to reduce pollution to the environment. The inventor was inspired to develop a process for extraction of bio-active compounds from such waste water not only for the sake of obtaining these bioactive compounds but also in order to increase the value of natural latex rubber while reducing
25 pollution in the environment, in alignment with Thailand's national strategy of using integrated Bio-Circular-Green (BCG) Economic Model to drive the country's economic and social development.

NRS contains dozens of biomolecules such as proteins and carbohydrates. [Wititsuwannakul D. & R. Wititsuwannakul. 2005. Biochemistry of natural rubber and structure of latex. in Steinbüchel, A. and Y. Doi (eds). 2005. *Biotechnology of Biopolymers: From Synthesis to Patents*. Chapter 4. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp. 73-123.] Even with minimal fractionation and purification, B-serum and C-serum fractionated by laboratory-scale centrifugation of *Hevea* latex have been shown *in vitro* to inhibit the proliferation of a breast cancer cell line (MCF7) [Lee, Y.K., L.K. Lay, M.S. Mahsufi, T.S. Guan, S. Elumalai and O.M. Thong. 2012. Anti-proliferation effect of *Hevea brasiliensis* latex B-serum on human breast epithelial cells. *Pak. J. Phram. Sci.* 25(3):645-650.] and that of liver carcinoma cells (Hep G2). [Lam, K.L., K.L. Yang, E. Sunderasan and M.T. Ong. 2012. Latex C-serum from *Hevea brasiliensis* induces non-apoptotic cell death in hepatocellular carcinoma cell line (HepG2). *Cell Prolif.* 45(6): 577-585.] Spray dried NRS, called “Natural Rubber Serum Powder” (NRSP), has been shown to comprise proteins, peptides, amino acids, and carbohydrates. NRSP has thus been used as the nutrient source for culturing micro-organisms such as *Zymomonas mobilis*, which produce ethanol. [Tripetchkul S., M. Tonokawa and A. Ishizaki. 1992. Ethanol production by *Zymomonas mobilis* using natural rubber waste as a nutritional source. *J. Ferment. Bioeng.* 74(6): 384-388]

Further purifications of NRSP have revealed bioactive peptides and nucleosides. NRSP has been shown to contain growth stimulating peptides that can stimulate the growth of *Bifidobacterium bifidum*, a probiotic. [Etoh, S., K. Asamura, A. Obu, K. Sonomoto and A. Ishizaki. 2000. Purification and identification of a growth-stimulating peptide for *Bifidobacterium bifidum* from natural rubber serum powder. *Biosci. Biotechnol. Biochem.* 64(10):2083-2088] NRSP has also been shown to contain 5'-methylthioadenosine (MTA), which can inhibit the growth of *P. falciparum* K1 (IC₅₀ 11.6 µM) while having no effect on Vero (African green monkey kidney, i.e. primate) cells. [Pitakpornpreecha, T., A. Plubrukarn and R. Wititsuwannakul. 2012. Quantification of 5'-deoxy-5'-methylthioadenosine in heat treated natural rubber latex serum. *Phytochem. Anal.* 23:12-15.] Furthermore, MTA has been shown to have anti-inflammatory properties, suppress the growth of *M. tuberculosis*, inhibit liver toxicity, and

halt the progress of certain cancers. [Suffrin, J.R., S.R. Meshnick, A.J. Spiess, J. Garofalo-Hannan, X-Q. Pan and C.J. Bacchi. 1995. Methionine recycling pathways and antimalarial drug design. *Antimicrob. Agents Chemother.* 39(11):2511-2515; Avila M.A., E.R. García-Trevijano, S.C. Lu, F.J. Corrales and J.M. Mato. 2004. Methylthioadenosine. *Int. J. Biochem. Cell Biol.* 36(11):2125-2130; Li, Y., Y. Wang and P. Wu. 2019. 5'-Methylthioadenosine and cancer: Old molecules, new understanding. *J. cancer.* 10(4):927-936.]

Besides peptides and nucleosides, quebrachitol, a monosaccharide, has been purified from NRS. [Udagawa, Y., M. Mashida, and S. Ogawa. 1991. *Method of Recovering L-quebrachitol from Rubber Latex Serums*. US Patent No. 5,041,689.] It is therefore not unexpected to find quebrachitol in NRSP. [Kiddle, J.J. 1995. Quebrachitol: A versatile building block in the construction of naturally occurring bioactive materials. *Chem. Rev.* 95:2189-2202.] Quebrachitol has been found to stop gastric ulcer, destroy free radicals, dissolve blood clots [Wang, D., S. Zhang, Z. Chang, D-X. Kong and Z. Zuo. 2017. Quebrachitol: Global status and basic research. *Nat. Prod. Bioprospect.* 7:113-122.], and promote the proliferation, differentiation and mineralization of osteoblast cells. [Yodthong, T., U. Kedjarune-Leggat, C. Smythe, R. Wititsuwannakul and T. Pitakpornprecha. 2018. L-Quebrachitol promotes the proliferation, differentiation, and mineralization of MC3T3-E1 cells: Involvement of the BMP-2/Runx2/MAPK/Wnt/ β -Catenin Signaling Pathway. *Molecules* 23(3086): 1-16.]

Oligosaccharides have also been fractionated from NRSP and found to inhibit the proliferation of Ca Ski cell line (cervical cancer of human papilloma viruses type 16 origin) in xenograph nude mice model and also inhibit the metastasis of cancer cells in mouse lung melanoma (B16-F1) metastasis model. [Wititsuwannakul, R. 2018. *Method for Preparing an Extract of Hevea Latex and Composition Thereof*. PCT International Patent Publication WO2018/044242 A1.]

Polysaccharides collectively is a class of biopolymers. Over 300 polysaccharides have been found in living organisms, especially in plants. Studies have shown the importance of polysaccharides in immune regulation, anti-inflammation, anti-virus, radio protection, hypoglycemic effects, and anti-neoplasm. [Yin, M., Y. Zhang and

- H. Li. 2019. Advances in research on immunoregulation of macrophages by plant polysaccharides. *Front. Immunol.* 10(145), 9 pp.] Polysaccharides have been purified and characterized from the juices of fruits and succulents such as *Aloe vera*, with the raw material for processing being similar to the waste water from the rubber industry, i.e.
- 5 diluted NRS with water as the main component. A polysaccharide-rich fraction from *Morinda citrifolia* fruit, with anti-tumor activity on lung carcinoma in mice, was found to contain pectic polysaccharides, homogalacturonan (4-GalAp), rhamnogalacturonan I (4-GalAp, 2-Rhap, 2,4-Rhap), arabinan (5-Araf, 3,5-Araf, t-Araf), type I arabinogalactan (4-Galp, 3,4-Galp, t-Araf) and β -glucosyl Yariv-binding type II arabinogalactan (3,6-Galp, t-
- 10 Araf). [Bui, A.K.T., A. Bacic and F. Pettolino. 2006. Polysaccharide composition of the fruit juice of *Morinda citrifolia* (Noni). *Phytochemistry*. 67:1271-1275.] Water-soluble polysaccharides which are components of *Aloe vera* gels, was found to promotes wound healing, alleviate the symptoms of skin cancer, psoriasis and eczema. [Chang, X-L., H. Xu, J-J. Wang, W-H. Wang and Y-M. Feng. 2013. Research on water soluble
- 15 polysaccharides separated from skin juice, gel juice and flower of *Aloe xerox* Miller. *Food Sci. Technol. Res.* 19(5), 901-907.] Water-extracted pectin from orange and other fruit juices yields polysaccharides that are useful as thickener, emulsifier, texturizer, and stabilizer in the food industry. In medicine, pectin is used as prebiotics and cholesterol controlling medication. [Dimopoulou, M., K. Alba, G. Campbell and V. Kontogiorgos.
- 20 2019. Pectin recovery and characterization from lemon juice waste streams. *J. Sci. Food Agric.* 99:6191–6198.]

Nevertheless, polysaccharides have not been isolated or characterized from NRS, NRSP, or effluent from the natural rubber manufacturing process. Calculated from Thailand's annual production of 14 million metric tons rubber latex serum, about 14

25 million liters of waste water is produced annually, requiring suitable treatments that increase the cost of the rubber. Failure to treat this wastewater would damage the environment, which is perhaps more expensive to remedy. A method for preparing polysaccharides from effluent from the natural rubber manufacturing process would thus help decrease the water pollution problem caused by rubber manufacturers while

30 providing a class of medicinally useful natural products.

Another goal that the present invention aims to achieve is the practicality of using the process in actual rubber manufacturing plants. Typically, a carbohydrate extraction technique from rubber latex in the prior art uses NRS as a starting material. Such a technique may work fine at the laboratory-scale but is not likely to be economically feasible when applied to the rubber manufacturing industry. The reason is that NRS generated in the laboratory relates directly to the liquid part of the natural rubber latex. In order to give the correct texture of the rubber product, however, the latex is routinely diluted with water in the factory before being mixed with diluted formic acid to start coagulation. In other words, the actual effluent that can be collected in the factory is much more diluted than the starting materials of a carbohydrate extraction processes that are published in research papers and patent documents. The volume of the effluent in comparison with the amount of carbohydrates present makes the entire extraction process economically unfeasible for extracting carbohydrates, including polysaccharides. What has been missing in the industry, so far, is a polysaccharide extraction process capable of using the actual effluent from a rubber manufacturing plant as its raw material.

It is an object of the present invention to prepare a polysaccharide extract.

It is an object of this invention to provide a method for preparing a polysaccharide extract from the rubber manufacturing plant effluent.

It is also an object of the present invention to provide a method for preparing such polysaccharide extract so that the polysaccharides are preserved, as much as possible, in their natural, native state.

It is also an object of the present invention to provide the method for preparing a polysaccharides extract from the rubber manufacturing plant effluent that uses low processing temperatures.

It is also an object of the present invention to provide the method for preparing a polysaccharides extract from the rubber manufacturing plant effluent that is energy-saving.

It is also an object of the present invention to provide the method for preparing a polysaccharides extract from the rubber manufacturing plant effluent that reduces waste arising from the rubber manufacturing process.

It is also an object of the present invention to provide a method for preparing such an extract or a composition comprising such an extract for use as a medicament.

It is also an object of the present invention to provide a method for preparing such an extract or composition for use in the treatment of inflammation.

5 It is also an object of the present invention to provide a method of using such an extract or composition in the manufacturing of a medicament for the treatment of inflammation.

Summary of the Invention

This invention is directed towards the extraction of polysaccharides from
10 rubber industrial process effluent.

One aspect of the disclosed invention is directed towards a method for extracting *Hevea* latex polysaccharides (HLPs) from the effluent of the rubber squeeze-milling process in the manufacturing of rubber latex products comprising a set of sequential steps: (a) providing rubber squeeze-milling process (rubber coagula calendering
15 process) effluent, which naturally comprises polysaccharides, as a starting material; (b) optionally removing debris and micro-organisms from such effluent, preferably by filtering; (c) concentrating the effluent by removing water from the effluent, preferably by an evaporation process; (d) further removing proteins from the effluent; (e) neutralizing the excess acidity, if any, of the deproteinized effluent; (f) precipitating polysaccharides
20 with an organic solvent; (g) collecting the precipitate; and (h) drying the precipitate to obtain the polysaccharide extract.

In another aspect of the invention, an extract prepared according to the said method is described.

Another aspect of the invention is a pharmaceutical composition comprising
25 such an extract and one or more pharmaceutically acceptable substances selected from the group consisting of additive, binder, carrier, diluent, excipient, filler, lubricant, solvent, and stabilizer.

Another aspect of the invention is directed towards such an extract or such a composition for use as a medicament.

Another aspect of the invention is directed towards such an extract or such a composition for use in the treatment of inflammation.

Another aspect of the invention is directed towards the use of such an extract or composition in the manufacture of a medicament for the treatment for inflammation.

5 The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

 These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the
10 accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such
15 substitutions, modifications, additions and/or rearrangements.

Brief Description of Drawings

 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The present invention may be better understood by reference to one or more of these drawings in combination with
20 the detailed description of specific embodiments presented herein.

 Figure 1 shows the process of extracting polysaccharides from process effluents in the Para (natural) rubber industry.

 Figure 2 shows a chromatogram of a polysaccharide extract. The resulting extract, after being dissolved in water, was analyzed using Size Exclusion-High-
25 Performance Liquid Chromatography (SEC-HPLC).

 Figure 3 shows the standard curve for molecular weight analysis of polysaccharide extract by SEC-HPLC.

Figure 4 shows the results of a proton Nuclear Magnetic Resonance (H^1 -NMR) analysis in deuterium (D_2O) solvent of polysaccharide extract obtained from the process according to this invention.

Figure 5 shows the toxicity of polysaccharide extract on lipopolysaccharides (LPS) stimulated RAW264.7 macrophages.

Figure 6 shows the anti-inflammatory effect of polysaccharide extract on LPS-stimulated RAW264.7 macrophages.

The figures and written description are not intended to limit the scope of the disclosed method for preparing the polysaccharide extract in any manner. Rather, the figures and written description are provided to illustrate the disclosed method for preparing the polysaccharide extract to a person having ordinary skill in the art by reference to particular embodiments of the invention. The invention will now be described with such particularity as to enable any person skilled in the pertinent art to practice the invention without extensive experimentation.

Detailed Description of the Invention

Hereinafter, the disclosure shall be described according to the preferred embodiments and by referring to the accompanying description and drawings. However, it is to be understood that referring the description to the preferred embodiments of the disclosure and to the drawings is merely to facilitate discussion of the various disclosed embodiments and it is envisioned that those skilled in the art may devise various modifications without departing from the scope of the appended claim.

As used herein, the terms “approximately” or “about”, in the context of concentrations of components, conditions, other measurement values, etc., means $\pm 5\%$ of the stated value, or $\pm 4\%$ of the stated value, or $\pm 3\%$ of the stated value, or $\pm 2\%$ of the stated value, or $\pm 1\%$ of the stated value, or $\pm 0.5\%$ of the stated value, or $\pm 0\%$ of the stated value. When the terms “approximately” or “about” is applied to a range of values, e.g. “about between 4 and 6 hours” the text should be understood as “between about 4 and about 6 hours”.

The process for extraction of *Hevea* Latex Polysaccharides (HLPs) from process effluents in the manufacturing industry of rubber latex products according to the present invention, as shown in Figure 1, comprises the following steps to be performed sequentially:

- 5 (a) *Providing rubber squeeze-milling process effluent, which naturally comprises polysaccharides, as a starting material.*

Referring to Figure 1, step 101 starts off the polysaccharide extraction process with the provision of the rubber squeeze-milling effluent. It should be emphasized that the starting material is the actual effluent that is obtained from a squeeze-mill of a rubber
10 manufacturing plant, not the latex serum that is prepared in the laboratory from natural latex.

- (b) *Optionally removing debris and micro-organisms from the effluent in the previous step, preferably by filtering.*

Step 102 comprises optionally removing debris, including micro-organisms
15 that contaminate the effluent during the rubber manufacturing process by filtering using a filter with a pore size (diameter) at the micron level. Initially the liquid is passed through a relatively larger pore size filter of approximately 30 μm to 1 μm in diameter in order to remove large particles. The liquid then goes through another filter with a finer pore size, about between 0.45 μm and 0.2 μm for removal of micro-organisms. Using coarse and
20 fine pore filters like these minimizes clogging and thus prolongs the service life of the fine filter at the expense of slower filtration rates. Using more than two levels of filtering is possible but unnecessary.

Removing debris and micro-organisms from the effluent in this step is not absolutely needed if the effluent is going to be concentrated (Step 103) on the same day it
25 is collected in Step 101 since the high temperature of the evaporation process helps deactivate most micro-organisms. If the effluent collected in Step 101 needs to be stored, e.g. in a liquid silo or holding tank, at least overnight before being concentrated in Step 103, Step 102 is needed to prevent polysaccharides from being broken down by micro-organisms. Removing debris and micro-organisms from the effluent helps keep the
30 evaporator in Step 103 clean. This is why Step 102 is placed at the front end of the whole

process, right after the effluent collection. This is also why this step starts with the word “optionally.”

(c) Concentrating the optionally filtered effluent in the previous step by removing water from the effluent, preferably by an evaporation process.

5 Step 103 comprises concentrating the filtered liquid from step 102. There are numerous ways to accomplish this step. Among the volume reduction techniques, thin-film evaporation is used to increase the concentration about between 5 and 30 times. This technique involves evaporation at a temperature about between 50°C and 100°C, preferably at about 100°C, thereby helping maintain polysaccharides in their natural,
10 native form as much as possible. Performing this step at higher temperatures may result in degradation of polysaccharides in the effluent.

The reduction in liquid volume in step 103 also helps eliminate proteins, as indicated in step 104. In principle, volume reduction reduces water activity, resulting in precipitation of the proteins. The precipitate is removed by filtration. In this invention,
15 step 104 is a by-product of step 103 without any additional energy cost or processing time. Persons skilled in the art may use other techniques to substitute for step 104.

(d) Further removing proteins from the effluent.

Although many proteins are removed in Step 104, in order to eliminate as much as possible the allergenic proteins that may remain in the effluent, Step 105 relies on
20 the biochemical principle of protein precipitation with acids. Many acids could be used here but the inventor found sugar acids or their derivatives to be effective for protein precipitation while being gentle to polysaccharides. A sugar acid is a monosaccharide with a carboxyl group at one end or each end of its chain, such as gluconic acid. The acid lowers the pH of the effluent to the point of proteins being denatured and eventually
25 precipitated out of solution.

The concentration of the sugar acid or its derivative is at least about 0.5% w/v of the liquid at a temperature about between 4°C and 100°C for a duration of about between 1 and 12 hours. Higher acid concentrations and higher temperatures contribute to more complete and faster precipitation of the proteins. Typically, precipitation is complete
30 in about 24 hours.

(e) Neutralizing the excess acidity, if any, of the deproteinized effluent.

The acidity of the supernatant from step 105 should be close to neutral, i.e. having the pH between 5 and 7. If the pH is found to be lower than this range after precipitating out proteins by an acid in step 105, step 106 comprises adjusting the pH to about between 5 and 7 with a base, preferably sodium hydroxide (NaOH). If the pH of the deproteinized effluent is found to be between 5 and 7, this step can be omitted.

(f) Precipitating polysaccharides from the effluent with an organic solvent.

Step 107 comprises precipitating out polysaccharides by treatment with an organic solvent at low temperature. In one embodiment, the organic solvent comprises about 95% v/v ethanol at about between 2 and 10 times the volume of liquid from Step 105 (in case no pH adjustment is needed) or from Step 106 (in case a pH adjustment is performed).

The preferred amount of ethanol is about between 4 and 6 times that of the solution volume at a temperature of about between -20°C and 4°C. The optimum incubation time for the precipitating reaction can be determined by a person of ordinary skill in the art. Nevertheless, it is wise to allow ample time, preferably about between 12 and 24 hours, for the precipitate to fully form. At lower temperatures, however, this time is reduced to about between 0.5 and 6 hours.

It is interesting to note that while polysaccharides are found in the precipitate, molecules like quebrachitol are found in the supernatant. In the prior art, quebrachitol is normally separated from other carbohydrates in a separate step. The inventor has found that the organic solvent precipitation step according to this invention separates quebrachitol and precipitates polysaccharides in a single step. This helps save cost and time in the extraction process.

(g) Collecting the precipitate.

The sticky, clumping sediment from step 107 can be collected in step 108 either by decantation or by centrifugation at about between 2,000 x g and 3,000 x g for a duration of about between 3 and 5 minutes. Decantation means the supernatant is discharged, poured out, poured off, drawn off, siphoned off, drained off, or otherwise transferred away from the precipitate or sediment. Conversely, the precipitate or sediment

may be removed from the supernatant. The exact embodiment of this step should be obvious to a person of ordinary skill in the art.

(h) Drying the precipitate to obtain the polysaccharides extract from the effluent of the rubber manufacturing process.

5 In one embodiment of this step, the precipitate collected in step 108 is dried by vacuum drying at a temperature about between 30°C and 100°C, preferably at about 60°C. The drying time depends on the temperature used. Higher temperature results in faster drying. Typically, it takes about between 6 and 12 hours to dry the precipitate, resulting in Extract 110, which comprises polysaccharides extracted from effluent obtained from
10 squeeze-milling of rubber coagula in the rubber manufacturing industry.

 Steps 102 to 109 inclusive, need to be practiced in this particular sequence. Persons skilled in the art, however, should be able to substitute and alter the details within each step. Arranging Steps 102 to 109, inclusive, in this particular sequence has a practical and logistical advantage when the method is practiced. Removal of debris and micro-
15 organisms by Step 102 helps reduce digestion of polysaccharides by micro-organisms that may be present in the starting effluent in Step 101. Step 103 reduces the liquid volume in order to concentrate the polysaccharides and reduce the overall processing time. This step is performed at a temperature high enough to aid evaporation but low enough not to break down polysaccharides into smaller sugar units. The by-product benefit of Step 103 is
20 proteins removal in Step 104. Further removal of proteins is achieved in Step 105, using a gentle precipitant in order to keep polysaccharides intact. After pH adjustments (if needed) in Step 106, the liquid is enriched in polysaccharides while devoid of most proteins. The polysaccharides are precipitated in Step 107, collected in Step 108, and dried in Step 109, again at relatively low temperatures in order to protect the integrity of
25 the carbohydrate polymers.

 From the above Detailed Description, it is important to note that no processing temperature ever exceeds about 100°C in the present invention. This helps preserve the polysaccharides in their natural state as much as possible.

Best Mode for Carrying Out the Invention

The Detailed Description of Invention mentioned above represents techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred mode and best mode for its practice. Nevertheless, changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Study of Preliminary Properties of the Polysaccharide Extract

Described below are representative test results. These results are presented to show the preliminary characterization and the preliminary safety test results of the *Hevea* latex extract according to the best mode of the present invention.

The extract from the present invention was shown to comprise carbohydrates by the phenol-sulfuric assay (Molisch's reaction). The assay is well-known in the field of carbohydrate biochemistry for over a century and is based on the dehydration of a carbohydrate by sulfuric acid to produce an aldehyde (either furfural or a derivative), which then condenses with a phenolic structure, such as phenol, resulting in a red or purple-colored compound.

Briefly, the extract was dissolved in deionized water (DI) and mixed with 5 %w/v phenol solution and concentrated sulfuric acid at a volume ratio of 1:1:5. The tubes were left at room temperature for 10 minutes, after which they were shaken and placed in a 25°C water bath for 20 minutes. The absorbance of the colored product was measured at 490 nm using a spectrophotometer (GENESYS™ 20 Visible absorbance spectrophotometer, Thermo Electron Scientific Corp., Madison, WI, USA) and read against a standard curve of sugar solutions. (Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers & F. Smith. 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* 28(3):350-356.) The data (not shown) confirmed the presence of carbohydrate(s) and gave the total carbohydrate concentration of the extract.

Figure 2 shows a chromatogram of the polysaccharide extract dissolved in water and being subjected to the analytical technique of SEC-HPLC. The horizontal axis represents the retention time, the length of time that a substance remains in the column.

The vertical axis represents the signal obtained from the Refractive Index Indicator (RID), a bulk property detector suitable for quantifying saccharides, which lack chromophores and therefore cannot be measured with a solute property indicating technique such as UV-Visible spectrophotometry. When a Polysep P-4000 size exclusion chromatography column with water as the mobile phase was used at the flow rate of 0.8 mL/min, the main signal (the major peak) was found between 10 and 12 minutes retention time. Although the tracing in Figure 2 is rather broad, consisting of overlapping peaks due to the heterogeneity and polydispersity of polysaccharides found in nature, the retention time of the major peak helps determine the retention volume for constructing the molecular weight standard curves shown in Figure 3.

Figure 3 shows the standard curve for molecular weight analysis of polysaccharide extract by SEC-HPLC with a Polysep P-4000 column. Water was used as the mobile phase with a flow rate of 0.8 mL/min. Two substances, dextran (Figure 3a) and polyethylene glycol (PEG, Figure 3b) of different molecular sizes were used in the preparation of these standard curves. The horizontal axes in both Figure 3a and Figure 3b represent the retention volume and the vertical axes in both figures show the log molecular weight of the substance used. The molecular sizes of the major peak, with respect to those of the dextran and the PEG standards, were found to be approximately 36.2 and 14.15 kDa, respectively. In other words, the molecular weights of most polysaccharide molecules in the extract, with respect to either standard, are above 10kDa. The two standards used in this measurement represent two extreme cases because the exact degree of branching of the polysaccharides in the extract was not known. On one hand, dextran comprises glucose monomers connected by β -1,6-glycosidic linkages in the main structure and by β -1,3-glycosidic linkages in the branches. On the other hand, PEG comprises unbranched ethylene glycol subunits.

Figure 4 shows the results of a ^1H -NMR analysis in D_2O solvent of polysaccharide extract obtained from the present invention. The horizontal axis represents the chemical shift while the vertical axis represents the signal intensity. The main signal in this Figure corresponds to that of polysaccharides. These substances contain sugars

with ^1H -NMR signals shifted less than 5 ppm upfield because the protons in the sugar structures are connected to carbons containing high electron densities (shielding effect).

In summary, (a) preliminary results obtained from carbohydrate determination using the phenol-sulfuric acid method indicate the presence of carbohydrates. (b) Figure 2 shows the heterogeneity and polydispersity of these carbohydrates in the extract. (c) Figure 4 shows that the carbohydrates are, in fact, polysaccharides. (d) Figure 3 shows that most polysaccharides in the extract are larger than about 10kDa, which translates to more than 20 sugar units. This is in contrast to monosaccharides and oligosaccharides in the prior art, that comprise no more than 20 sugar units and are smaller than 10kDa.

From the preliminary properties summarized above, this present invention has therefore accomplished its objects of preparing a polysaccharide extract from the rubber manufacturing plant effluent in such a way that the polysaccharides are preserved in their natural, native state as much as possible, i.e. with minimal cleavage or hydrolysis that would decrease their molecular weights. This is achieved by keeping the temperature in each step not higher than about 100°C. This relatively low processing temperature also imparts energy-saving benefit to the manufacturing plant, compared to other effluent treatment processes with similar capacity.

It is also evident that the process according to the present invention is environmental-friendly. The use of low temperature in the extraction process is energy saving. All water used in the process comes from the raw material, i.e. the effluent. The water saving and utilization of effluent both contribute to preserving the environment.

The method according to the present invention produces by-products which can be further utilized. Separation of polysaccharides from the effluent, according to the present invention, is performed without the need to first separate out small carbohydrates, like quebrachitol, which remain in the supernatant in the organic solvent precipitation step. Such supernatant may be collected and used as a source for extracting these mono- and oligosaccharides. The proteins removed in the acid precipitation step can be used as animal feed, an organic fertilizer raw material, a protein source for cell culture in an ethanol production process, etc.

Preliminary study on the toxicity and anti-inflammatory activity
of the polysaccharide extract

Studies in the toxicity and anti-inflammatory activity of the polysaccharide extract on RAW264.7 macrophages were performed by first dissolving RPMI-1640 instant cell culture medium - powder type (Gibco™, USA) in deionized water according to the manufacturer's recommendations. Then, sodium bicarbonate (NaHCO₃) was added at 2 g/L of the cell culture medium. The pH was adjusted to 7.2 with concentrated hydrochloric acid. Then heat-inactivated Fetal Bovine Serum (Gibco™ USA) and Penicillin-Streptomycin Solution (Gibco™ USA) were added to make the final concentrations 10% and 1%, respectively. After that the prepared complete medium was sterilized and filtered through a 0.2 micron membrane and stored at 4 °C.

Macrophages (RAW264.7, CLS Cell Lines Service GmbH, Germany) were plated on 96-well plates at 1×10^5 cells per well. The plates were incubated at 37 °C under 5% carbon dioxide atmosphere. At the end of a 24-hour incubation, the cell culture medium was aspirated from each well. A 100 µL aliquot per well of polysaccharide extract at 12.5, 25, 50 and 100 µg/mL in complete medium free of phenol red was then added. Cells were stimulated by additions of 100 µL per well lipopolysaccharide from *E. coli* (Sigma, USA), making the final concentration of 1 µg/mL. Incubation was repeated under 5% carbon dioxide for another 24 hours at 37°C.

The toxicity test of polysaccharide extract on LPS-stimulated RAW264.7 macrophages was performed in comparison with a control group that had only RPMI cell culture medium and another control group that had been treated only with LPS. The test was initiated by adding 10 µL Cell Proliferation Reagent WST-1 (Roche, Switzerland) to each well containing 100 µL of cell culture and then incubated at 37 °C for 1 hour. The absorbance was then measured at 450 nm. Cellular toxicity could be assessed from the cell's survival rate, which was calculated from the equation below:

$$\text{Survival rate (\%)} = (450\text{nm absorbance of sample} / 450\text{nm absorbance of control}) \times 100.$$

Results of the study, shown in Figure 5, indicate that the polysaccharide extract, at 12.5, 25, 50 and 100 µg/mL, was not only non-toxic but also growth-promoting

at low concentrations. (An asterisk above a bar graph indicates that the result for the particular condition is statistically significant compared to control.) In RAW264.7 macrophages, the polysaccharide extract, at low concentrations of 12.5 and 25 µg/mL, was found to have significant growth-promoting effect. The aspirin standard (200 µg/mL), a positive control, was found to be non-toxic but without any growth promoting effect. This concentration of aspirin standard was about double the reported plasma level, 30 to 100 µg/mL, of aspirin for regular therapeutic doses. [Arif, H and S. Aggarwal. 2022. Salicylic Acid (Aspirin). StatPearls (Internet). National Center for Biotechnology Information, National Library of Medicine, National Institute of Health.]

Studies on anti-inflammatory activities of the polysaccharide extract on RAW264.7 macrophages were performed in the same way as the toxicity test described above. At the end of the incubation, 100 µL of cell culture medium in each well was transferred to a new 96-well plate, and 100 µL of Griess reagent (Merck, USA) was added to each well. The plate was incubated at room temperature and protected from light for 15 min. Absorbance was measured at 548 nm. Nitric oxide (NO) produced by macrophages were read against the standard curve of sodium nitrite (NaNO₂).

Results of the study, shown in Figure 6, indicate that the polysaccharide extract at concentrations of 12.5, 25, 50 and 100 µg/mL, significantly and in a dose-dependent manner, inhibited the secretion of NO, an inflammatory mediator in LPS-stimulated RAW264.7 macrophages. The inhibitory effect of polysaccharide extract at 100 µg/mL was comparable to that of the standard aspirin (positive control) which was at a higher concentration of 200 µg/mL. At the same concentration, the polysaccharide extract was about twice as effective as aspirin in inhibiting NO secretion.

The anti-inflammation property of the polysaccharide extract should bestow it to be used as a medicament in the treatment of inflammation, and to be used in the manufacturing of a medicament for the treatment of inflammation. The polysaccharide extract according to the present invention can also be combined, to form pharmaceutical compositions, with one or more pharmaceutically acceptable substances selected from the group consisting of additive, binder, carrier, diluent, excipient, filler, lubricant, solvent, and stabilizer. Such compositions can also be used as a medicament in the treatment of

inflammation, and to be used in the manufacturing of a medicament for the treatment of inflammation.

This present invention has therefore accomplished its objects of preparing a polysaccharide extract from the rubber manufacturing plant effluent, for use as a medicament in the treatment of inflammation, and for use in the manufacturing of a medicament for the treatment of inflammation.

Starting from the desire to protect the environment and minimize the cost of natural rubber production, the inventor has unexpectedly discovered that the method according to the detailed (and the best mode) of the present invention gives the high-molecular weight, presumably native and unhydrolyzed or minimally hydrolyzed, form of polysaccharides. It is the first time that polysaccharides with molecular weights larger than about 10 kDa can be isolated directly from the effluent. In the prior art, NRS is usually pre-dried using the technique of spray drying at high temperature to produce natural rubber serum powder (NRSP), which is a source of proteins, peptides, and amino acids. NRSP is the usual raw material for extraction of small-molecule carbohydrates like oligosaccharides, and especially quebrachitol.

Finally, it should be emphasized that the exact chemical nature of the high molecular weight carbohydrates according to the present invention is still under investigation. It is likely that the exact composition of the extract may vary depending on the starting material, e.g. the rubber latex, and the latex collection process. Nevertheless, the extract according to the present invention has shown an anti-inflammatory activity while being non-toxic to cell cultures.

Applicability to the Pharmaceutical Industry

From the anti-inflammatory effect of the extract, it is obvious that the invention is applicable to the pharmaceutical industry. The applicant itself is in the process of sponsoring studies aimed at pharmaceutical development of the extract and/or a composition comprising such extract and one or more pharmaceutically acceptable substances into an anti-inflammatory medicament for humans and animals. This invention is exceptional in that it is expected to have both economic impacts from the business

standpoint as well as social impacts from decreasing the enormous volume of effluent generated by the rubber industry.

CLAIMS

1. A method for extracting *Hevea* latex polysaccharides from effluent of the rubber squeeze-milling process in the manufacturing of rubber latex products, the method comprising steps to be performed sequentially:
 - 5 (a) providing rubber squeeze-milling process effluent, which naturally comprises polysaccharides, as a starting material;
 - (b) optionally removing debris and micro-organisms from such effluent, preferably by filtering;
 - (c) concentrating the effluent by removing water from the effluent,
10 preferably by an evaporation process;
 - (d) further removing proteins from the effluent;
 - (e) neutralizing the excess acidity, if any, of the deproteinized effluent;
 - (f) precipitating polysaccharides with an organic solvent;
 - (g) collecting the precipitate; and
15 (h) drying the precipitate to obtain the polysaccharide extract.
2. The method according to claim 1, wherein each of the sequential steps is performed at a temperature not higher than about 100°C.
3. The method according to any one of claims 1 to 2 inclusive, wherein the filtering technique for optionally removal of debris and micro-organism comprises filtration
20 through a filter with micron-level pore size (micro filtration).
4. The method according to any one of claims 1 to 3 inclusive, wherein the filtering technique for optionally removal of debris and micro-organisms comprises filtration through a series of filters with pore diameters of about 30 µm down to about 0.2 µm.
- 25 5. The method according to any one of claims 1 to 4 inclusive, wherein the concentrating technique by evaporating out water comprises the technique of thin-film evaporation.
6. The method according to claim 5, wherein the temperature used in the thin-film evaporation technique is in the range about between 50°C and 100°C inclusive,
30 preferably at about 100°C.

7. The method according to any one of claims 1 to 6 inclusive, wherein the further protein removal step comprises precipitating proteins by an acid, preferably by a sugar acid or its derivative.
8. The method according to claims 7, wherein the sugar acid is preferably gluconic acid.
9. The method according to any one of claims 7 to 8 inclusive, wherein the concentration of the sugar acid or its derivative is about between 10 and 30 per cent weight/volume.
10. The method according to any one of claims 1 to 9 inclusive, wherein the further protein removal step is performed at a temperature about between 4°C and 100°C for a duration of approximately between 1 and 12 hours.
11. The method according to any one of claims 1 to 10 inclusive wherein the neutralization of excess acidity of the deproteinized effluent, if any, is performed by adjusting the pH of the deproteinized effluent to approximately between pH 5 and 7.
12. The method according to any one of claims 1 to 11 inclusive, wherein the pH of the deproteinized effluent is adjusted by a base, preferably sodium hydroxide.
13. The method according to any one of claims 1 to 12 inclusive, wherein the organic solvent used for precipitating out polysaccharides comprises ethanol.
14. The method according to claim 13, wherein the ethanol used for precipitating out polysaccharides comprises at least about 95% ethanol.
15. The method according to any one of claims 13 to 14 inclusive, wherein the ethanol used for precipitating out polysaccharides is used at a volume about between 2 and 10 times the volume of the deproteinized effluent.
16. The method according to any one of claims 1 to 15 inclusive, wherein the temperature used in the polysaccharide precipitation step is approximately between -20°C and 4°C, preferably at about 4°C, for a duration of about between 0.5 and 24 hours.

17. The method according to any one of claims 1 to 16 inclusive, wherein the precipitation is collected by decantation, or by centrifugation at about between 2,000 x g and 3,000 x g for a duration of about between 3 and 5 minutes.
18. The method according to any one of claims 1 to 17 inclusive, wherein the drying
5 step comprises oven-dry under vacuum.
19. The method according to any one of claims 1 to 18 inclusive, wherein the drying temperature is about between 30 and 100°C, preferably at about 60°C.
20. The method according to any one of claims 1 to 19 inclusive, wherein drying is done under vacuum of below about 0.001 MegaPascal (MPa).
- 10 21. An extract prepared according to any one of claims 1 to 20 inclusive.
22. A pharmaceutical composition comprising the extract prepared according to any one of claims 1 to 20 inclusive and one or more pharmaceutically acceptable substances selected from the group consisting of additive, binder, carrier, diluent, excipient, filler, lubricant, solvent, and stabilizer.
- 15 23. The extract according to claim 21 or the composition according to claim 22 for use as a medicament.
24. The extract according to claim 21 or the composition comprising such extract according to claim 22 for use in the treatment of inflammation.
- 20 25. Use of the extract according to claim 21 or the composition comprising such extract according to claim 22 in the manufacture of a medicament for the treatment of inflammation.