FUNGAL RESISTANCE AND LEACHABILITY OF GENIPIN-CROSSLINKED CHITOSAN TREATED WOOD

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Abstract

As a natural antimicrobial compound, chitosan (C) has been widely explored for wood protection against numerous deteriorating organisms and has proven its effectiveness. However, the application of chitosan as a biobased preservative has been significantly limited due to its high leachability from treated wood. Genipin is a biobased crosslinking agent that can crosslink with chitosan in very mild conditions. The objective of this study was to examine the decay resistance and leachability of genipin-crosslinked chitosan (GC) treated wood against common wood-decaying fungi. The formation of GC was confirmed using Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA). The antifungal efficacy of GC against two brown-rot fungi, *Gloeophyllum trabeum* (*G.t.*) and *Rhodonia placenta (R.p.)* and two white-rot fungi, *Trametes versicolor (T.v.)* and *Irpex lacteus (I.l.)* was first determined using malt-gar as substrate, which revealed that GC inhibited the growth of all fungi studied and exhibited similar efficacy to those of C treatment levels. Light and fluorescence microscopy showed changes in fungi morphology and nuclei deformation due to the effect of GC. Upon impregnation, GC-treated wood samples show an increased retention and mass gain as the function of treating concentrations, which were as high as 21 kg/m^2 and 3.6%, representatively. However, cross-linking chitosan with genipin did not reduce the leaching rate of chitosan. GC treated wood samples generally show a significantly lower mass loss than those of the control groups regardless of the leaching test.

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Dedication

I dedicate this work to the Almighty God, my parent; Mr. and Mrs. Alorbu and to all my siblings Mary-an, Emmanuella and Prince.

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List of Abbreviations and Acronyms

ACQ	Alkaline copper quat
ACZA	Ammoniacal Copper Zinc Arsenate
ANOVA	Analysis of Variance
AWPA	American Wood Protection Association
C/CH	Chitosan
CA	Copper azole
CCA	Chromated Copper Arsenate
CCC	Chitosan Copper Complex
DA	Degree of acetylation
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DTG	Derivative Thermogravimetry
FRE	Flame Retardant Efficiency
FTIR	Fourier-Transform Infrared
GC	Genipin Crosslinked Chitosan
HDPE	High Density Polyethylene
HMW	High Molecular Weight
KOH	Potassium hydroxide
LMW	Low Molecular Weight
MCA	Micronized copper azole
MDF	Medium Density Fiber Board
MOE	Modulus of Elasticity
OCB	Oriented Cotton Stalk Board
PVA	Polyvinyl Alcohol
SEM	Scanning
TGA	Thermogravimetric Analysis
TPP	sodium tripolyphosphate
WPC	Wood Plastic Composite

Chapter 1: Introduction

1.1 Background

Wood is a versatile and renewable building material that has been widely used in both outdoor and indoor applications. It is one of the oldest commonly used building materials by human that has always been derived from the trunk consisting of elongated cells with unusual strength, flexibility and durability at both macroscopic and ultrastructure levels [1], [2]. However, due to the chemical structural makeup of wood, it is known to be susceptible to deterioration by various organisms, such as fungi, insects, termites, and marine borers, when exposed to high moisture environment for a prolonged time [3], [4]. Therefore, to extend the service life of wood and wood products, the material must be protected before putting into service. Over the years, the use of naturally durable wood species such as red wood and cedars has been an option to achieving an appreciable service life of wood in service, but these wood species are not in large quantities compared to the moderately and less durable wood species and hence cannot meet the increasing demand levels. Therefore, chemical preservation of wood has been the effective way of protecting wood from these degrading organisms.

In recent times, copper-based chemicals such as chromated copper arsenate (CCA) and micronized copper azole (MCA) is among the commonly used water-borne preservatives being used to treat wood especially for residential and commercial applications [5], [6]. Despite their high effectiveness in protecting wood against wood deteriorating agents, there are rising concerns of the potential toxicity of these chemicals (i.e., CCA) to aquatic organisms and the disposal issues of chemically treated wood [7]. Moreover, some brown rot fungi are reported to have developed tolerance to these synthetic wood protectants[8]. These drawbacks have necessitated the need of developing new wood preservative formulations that are not only effective against wood decay but also less harmful to the environment.

One of the most promising solutions is the use of natural preservatives for wood protection. In recent years, numerous research studies have been conducted investigating the efficacy of plants and animal extracts as natural preservatives for wood protection including plant extracts of essential oils, extractives, and tannins [9]–[11], fungal extracts[12] and natural

antimicrobial polymers [13]. One of the natural polymers that gained lots of attention for wood protection is chitosan [14] because it is bioactive, renewable, biodegradable and relatively inexpensive [15].

Chitosan is a linear polysaccharide that consists of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) units [16]. It is extracted and converted from chitin, the second-most abundant natural biopolymer presents in the exoskeleton of crustaceans, cell walls of some fungi and algae, through alkaline deacetylation [17]. The antimicrobial properties of chitosan against wood decay fungi [10], [18]–[25], mold and stains [26]–[28] and termites [22], [29]–[32] has also been extensively investigated over the past two decades. In these studies, chitosan has been reported to be effective in inhibiting the growth of fungi (fungistatic) and may also act as a fungicide (fungitoxic) at a higher concentration [17], [19]. Additionally, chitosan with a high average molecular weight appeared to be more effective against mold and staining fungi than chitosan with a low average molecular weight [18]. Despite the promising results of using chitosan for wood protection from the numerous studies, the intrinsic hydrophilicity of chitosan and the high leachability of chitosan-treated wood when exposed to elevated moisture environment remains a significant drawback that has affected the patronization and utilization of the biopolymer [18], [31].

One of the efforts that has been attempted was using high molecular weight (HMW) (215 kDa) chitosan for wood treatment. Although the HMW chitosan was more retained in wood upon leaching than low molecular weight (LMW) (35 kDa) chitosan, the initial retention of these HMW chitosan molecules in wood is relatively low, thus providing limited protection efficacy [21], [33]. Other researchers have also worked on cross-linking chitosan with glutaraldehyde to endow it with a networked structure and to improve its water resistance in fiberboard, but the durability of the wood products after leaching was not investigated [9], [34], [35]. Moreover, the high toxicity of this synthetic cross-linker, glutaraldehyde, is still a big concern.

On the other hand, genipin is a natural cross-linker of low-toxicity derived from the fruits of *Genipa americana* and *Gardenia jasminoides Ellis* [36]. It was proven to be 5000-10,000 times less toxic than glutaraldehyde [37]. Genipin can also react with the amine groups of

biopolymers such as chitosan, resulting in a polymeric network with enhanced thermal and operational stabilities as well as antimicrobial properties [38], [39]. Genipin crosslinked chitosan (GC) has been extensively studied in tissue engineering [40], drug delivery [41] and food processing industry[36]. However, no studies have been reported using genipin-crosslinked chitosan formulation for wood protection. Therefore, based on the superior properties of GC that has been established in previous studies and the low leachability of HMW chitosan with improved biological activity against wood decay organisms, we hypothesize that GC will be as effective as unmodified chitosan in terms of the efficacy against wood decay fungi and will be more stable in treated wood thereby reducing the high leaching prowess of chitosan.

1.2 Research Objectives

The overall objective of this study seeks to examine the decay resistance and leachability of genipin crosslinked chitosan (GC) treated wood against two brown rot fungi; *Gloeophyllum trabeum* (*G.t.*) and *Rhodonia placenta (R.p.)* and two white-rot fungi: *Trametes versicolor (T.v.)* and *Irpex lacteus (I.l.)*.

To achieve these goals, the antifungal properties of genipin crosslinked chitosan (GC) against two brown-rot fungi; *Gloeophyllum trabeum* (*G.t.*) and *Rhodonia placenta (R.p.)* and two white-rot fungi: *Trametes versicolor (T.v.)* and *Irpex lacteus (I.l.)* in an *in vitro* test will be examined. The effect of GC on fungi morphology was also examined using light and fluorescence microscopy. Instead of treating wood with GC directly, a fresh GC precursor solution was prepared by mixing genipin with low molecular weight (LMW) chitosan, after which was used to treat wood samples and the cross-linking reaction of GC within the wood microstructure was induced by oven-drying at 60 ^{o}C for 48 h. The formation of GC was confirmed using Fourier Transform Infrared (FTIR) spectroscopy and Thermogravimetric analysis (TGA) characterization methods. Quantification of GC in wood after treatment and after leaching test was also reported. The biological efficacy of GC treated wood against brown rot and white rot fungi is also presented.

Chapter 2: Literature Review

2.1 Wood as a natural resource

Wood is a renewable material of great value and importance to the world economy. It is one of the most utilized resources around the globe including but not limited as a structural material, fuel, and as an industrial raw material from which other products can be produced. It is a natural resource sourced from the forest which is estimated to cover about one-third of the land areas of the world. Wood is available in large quantities at comparatively low cost and its production can be increased through sustainable forest management practices [1]. In the United States, over 100 species of wood are available in the forest areas out of which 60% are of major commercial importance. Some major positive characteristics of wood as a green material includes low embodied energy, low carbon impact and a highly sustainable resource [42], [43]. Botanically, there are two main classifications of wood: softwoods and hardwoods. Harwood are derived from angiosperms and are deciduous broad-leaved trees whiles softwoods are from gymnosperms and are generally coniferous trees with needle-like shaped leaves[42]–[44]. These two main classifications of wood differ anatomically, chemically, and mechanically.

2.1.1 Structural components of wood cell wall

The plant cell wall is a complex biological structure that performs numerous functions throughout the lifespan of the plant (Figure 2.1). It is also made up of wide range of channels, pores and receptors that regulate movement of molecules and responses elicitors including hormones, sugars, and proteins [45], [46] The cell wall is an important component of woody plants because it determines the major properties of the wood. Wood cell walls consist of three main layers: a middle lamella, a primary cell wall, and a secondary cell wall that differ in the timing of their synthesis and their chemical composition. The middle lamella is the lignified outermost layer that serves as the adhesive that binds adjacent wood cells together [42] The primary cell wall is the thin layer (unlignified) that is in contact with the middle lamella and is mainly composed of cellulose, hemicelluse and pectin[47]. The secondary cell wall is attributed to constitute most of the mass of wood cell walls and is deposited at a later stage when the cell has stopped growing and dividing. It is the thickest, lignified and most

durable amongst the three walls and woody plants heavily relies on the secondary wall for mechanical support [45], [48], [49]. The secondary cell wall is the point of refence for wood decay fungi during deterioration for outmost mechanical destruction. It is important to note that all the cell walls are modified with perforations called pits that allow communication and transport of between cells in livings plants. Pit size, type and relative propertions varies with wood species and greatly determines the behavoir of wood in various situations such as how wood interacts with surface coating [42], [50].

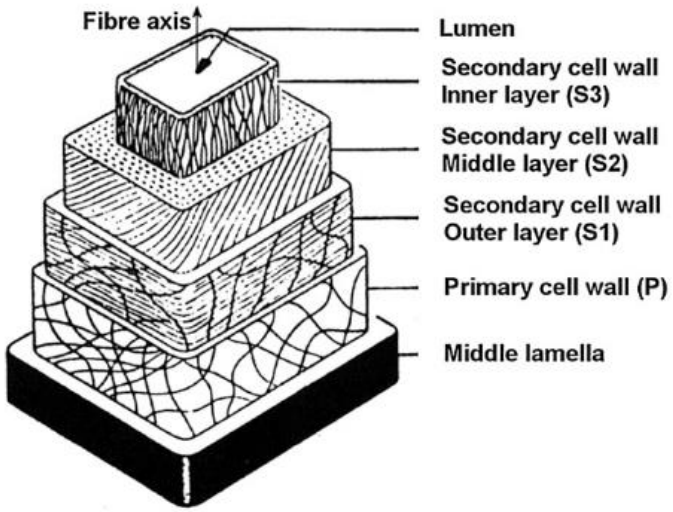

Figure 2. 1 Structural make of wood cell wall [51]

2.1.2 Chemical compositions of wood

The major chemical component of cells in living trees is water, but on a dry weight basis, wood mostly consist of 65-70% carbohydrates that are bonded together by 18-34% lignin [47], [52]. Other minor components of wood include organic extractives and inorganic minerals (ash) (4-10%). The carbohydrates components of wood mainly comprise of cellulose and hemicellulose and on dry wood basis, the formal ranges from 40-50% whiles the latter 25-35% [52], [53] According to Ruiz-Aquino et al. [54] , wood varies within and between tree species, geographic location, and growth conditions thereby rendering the precise definition of chemical composition wood for a particular of group of species unachievable.

2.1.2.1 Cellulose

Cellulose, known to be the most abundant organic chemical on earth, is the major chemical component the secondary cell wall of wood comprising of 40-55% of dry wood weight (Figure 2.2) [45], [48]. It is a polysaccharide composed of glucopyranose units linked by β-1-4 linkages to form high molecular weight linear polymer, with each monomer unit alternating at 180 ° to the other [55]. The number of glucose unit in cellulose defines its degree of polymerization which on the average have been determined to be 10,000 for cellulose. Cellulose can form intra- and inter-molecular hydrogen bond on the linear cellulose chains due to the abundant hydroxyl groups in its glucose units. These hydrogen bonds bind single cellulose chain units together as an aggregate called cellulose microfibrils [52]. The packing density of cellulose units greatly affects its properties- an increased packing density leads to the formation of crystalline regions whiles a lower packing density leads to the formation of amorphous regions (Figure 2.3) [53].

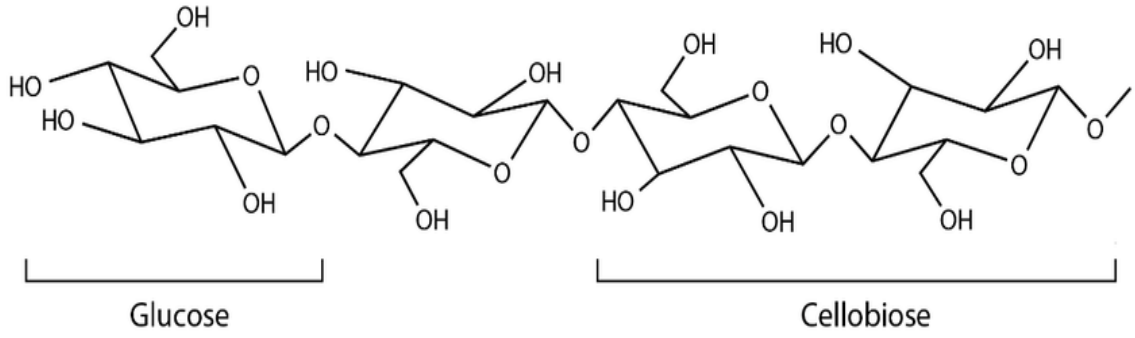

Figure 2.2 Molecular structure of Cellulose[56]

Cellulose in wood is mostly crystalline (65%) in nature making it unreadily accessible to water and microorganisms. This explains the high tensile properties of wood and woody materials. However, cellulose in wood also contains amorphous region (35%) that readily accessible to water and microorganisms and is mostly attributed for the hydrophilic properties of wood due to the presence of the hydroxyl groups that rapidly bonds with water. This is responsible for excellent adhering surfaces of wood [53]. The amorphous region of cellulose bundles also allows the penetration of microorganisms such as fungi hyphae to cause the degradation of wood assuming conditions are favorable for decay[10].

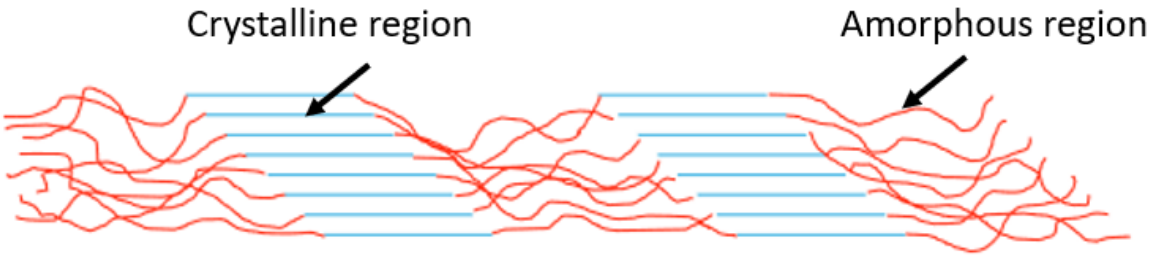

Figure 2. 3**:** Depicting the crystalline and amorphous regions of cellulose [57].

2.1.2.2 Hemicellulose

Hemicellulose is another polysaccharide present in the secondary cell walls of wood. It consists of a group of polymers with a lower degree of polymerization (average DP of 100–200) than cellulose and mainly contains the sugars D-xylo-pyranose, D-glucopyranose, D-galactopyranose, L-arabinofuranose, D-mannopyranose, D-glucopyranosyluronic acid, and D-galactopyranosyluronic acid with minor amounts of other sugars[47]. However, there are two major components of hemicellulose based on the two classifications of wood: xylans in hardwood and glucomannans in softwoods hence requires different enzymes to break them down [52]. The hemicellulose is usually encrusted among the cellulose microfibril bundles and are sometimes difficult to differentiate but together, they contribute to the structural components of the standing tree. Hemicellulose is prone to biological deterioration because of the attached acid residue that are susceptible to the intake of water [47], [58].

Other minor chemical present in both softwood and hardwood includes pectin, proteins, and starch [58]. Pectin are highly complex polysaccharides that is major component of the primary cell wall of hardwood species, but it is also present in small quantities in cell walls of both softwood and hardwoods [59] They are also found in the membranes in the boarded pits between wood cells and in the middle lamella and degradation of these membranes by microorganisms will increase the permeability of preservative treatment solutions. Starch on the other hand serves as the storage unit of the polysaccharides produced by plants during growth found in parenchyma cells [47].

2.1.2.3 Lignin

Lignin in wood constitute 22-33% of the dry wood weight and is known as the cementing substance in wood cells. It is a relatively complex substance that is the product of enzymatic dehydrogenative polymerization of three phenylpropanoid monomers namely coniferyl, sinapyl and *p*-couumaryl alcohols (Figure 2.4) [60]. Softwood contained lignin that are known as guaiacyl lignins because coniferyl alcohol constitutes more than 90% of the structural elements and the remaining elements being the *p*-coumeryl alcohol type. On the other hand, harwood contained lignin are termed guaiacyl–syringyl lignins, because they are composed of both coniferyl and synapyl alcohol types in varying proportions. In general, lignin found in wood is distributed throughout the secondary cell wall with the highest concentration in the middle lamella and the primary wall region. The two major functions of lignin in wood are for binding and stiffening wood fibers through its distribution between and within the cell walls [58], [60], [61].

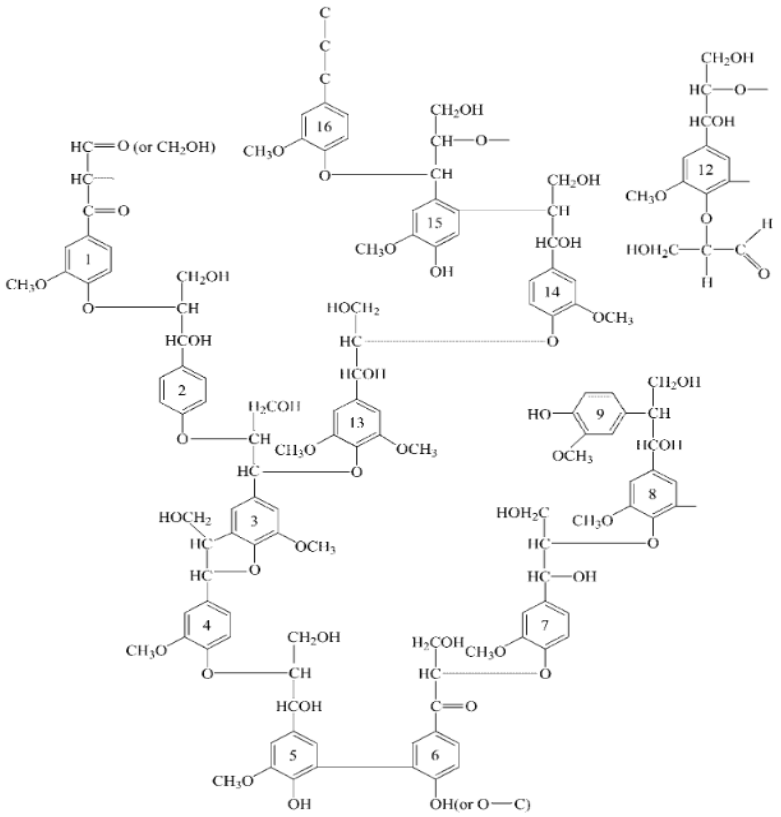

Figure 2. 4**:** Chemical structure of softwood Lignin [60]

2.1.3 Major applications of wood

Wood as a relatively abundant construction material that is available in wide range of texture, color, densities, and chemical compositions and has been used in numerous applications over the years. Some major applications that wood includes timber and lumber for construction purposes, veneer for decorative paneling, plywood, piling and wharves, railroad ties, poles and posts, packaging and crates, paper and its related products, cellulose derivatives, charcoal and many more[1]. However, wood usage in the 21st century is driven by social, economic, and environmental factors that managers and decision makers of the forestry sector cannot control [61]. Some of these factors include expanding world population with repelling effect on energy, shelter, food, and the need to adapt to shifting demographic patterns, rapid technological changes, and climate change. Forestry managers and policy makers must take drastic decision with focus on efforts in silviculture, tree genetics, tree propagation, and tree productivity to substantially and sustainably increase both the yields and quality of wood to meet the growing population and its repercussive factors [1], [61].

2.2 Wood decay and its characteristics

Despite the numerous advantages of wood as a construction material, the material can be degraded by biological deteriorating organisms with the appropriate conditions available leading to a drastic reduction in mechanical and aesthetic values of it. Some of these wood deteriorating agents of wood includes fungi, termites, molds, stains, marine borers and to a mild extent bacteria’s depending on the areas of application of wood. It is important to note that other abiotic factors including but not limited to fire, weathering and mechanical wear also cause the degradation of wood[1], [62].

Decay in wood is largely caused by fungi which are classified in two main categories; brown rot and white rot fungi, based on the appearance of the degraded wood which goes a long way to tell the polymeric materials that were degraded. The polymeric materials in wood that are of target for the decay fungi are the cellulose, hemicellulose, and lignin. Brown rot fungi during decay selectively targets the carbohydrates (cellulose and hemicellulose) for digestion leaving brown colored lignin rich wood residue whiles the white rot fungi are characterized

by their ability to degrade the lignin in addition to the cellulose and hemicellulose portions of wood [1], [10], [62].

However, it is important to note that fungi just as any living organisms requires conditions for growth and development and the five most important requirements for fungi to grow in wood includes availability of free water (~30%), air, temperature (ranges between 10 and 32 °C), food substrate (wood) and source of fungi infection or spores[10], [63]. Therefore, to prevent fungi infestation in wood, at least one of these conditions and requirements must be absent or deliberately removed.

2.2.1 Brown rot

Wood colonized by brown rot fungi aim for the carbohydrate components in the cell wall of the material even though all cell wall components are affected. Brown rot fungi mostly attack softwood species which the bulk of the construction timber materials in North America and the European continent and as such is of critical importance to the wood user. Brown rot as after extracting the carbohydrate component of the wood leaves a brownish, fractured lignin rich residues (Figure 2.5) [10]. According to Schilling et al. [64], brown rot fungi during wood decay uses the reactive oxygen species (ROS) mechanism to loosen the cell wall components of the wood before selectively extracting the carbohydrates using the glycosyl hydrolases carbohydrate enzymes. Even though brown-rot fungi have been reported to degrade low lignin polymers, recent studies show some brown rot fungi species can cause the removal of up to 40% lignin from corn stover, pine and aspen wood[65], [66] This type of decay results in drastic changes in timber properties such as tension or bending even with very low mass loss [67]. Some of the most common brown rot fungi that have been reported to cause significant loss in conifer wood properties due to decay includes *Neolentinus lepideus, G.t., G. sepiarium, Rhodonia (Poria monticola) placenta, Meruliporia (Poria) incrassata, Coniophora arida var. suffocata, Fibroporia (Poria) vaillantii, Antrodia (Poria) xantha, Coniophora puteana, and Fibroporia (Poria) radiculosa*[1].

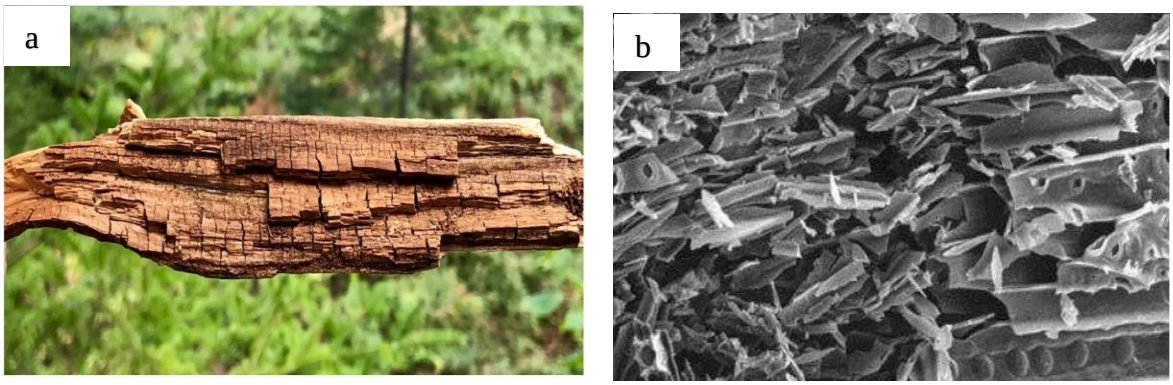

Figure 2. 5**:** a) Photograph of brown rot wood residue and b) SEM micrograph of wood after brown rot fungi decay in softwood [62], [68]

2.2.2 White rot

White rot fungi are numerous and the most common species wood decay fungi and unlike brown rots, white rot fungi can degrade lignin [68]. There are two types of white rot fungi namely simultaneous and selective lignin degrading fungi. Simultaneous white rot fungi degrade all three components of the cell wall whiles utilizing them whiles the selective white rot fungi preferentially target and degrade the hemicellulose and lignin portions of the cell wall whiles leaving the crystalline cellulose undegraded (Figure 2.6b) [62], [68]. Nonetheless, both white rot types degrade lignin using oxidative enzymes such as laccase, lignin peroxidase, manganese peroxidase, versatile peroxidase and the dye-decolorizing peroxidases whiles gaining access to the carbohydrate active enzymes (CAZYs) such as glycosyl hydrolases which then target the hemicellulose and cellulose portions [1], [10], [64], [68]. White rot fungi mostly prefer degrading hardwoods and structural loss during decay is usually slow compared to brown rot fungi. Wood mass leftover after decay appears bleached, spongy, and light in weight with flecks of white mycelium mat embedded in the wood fibers when decay is in advanced stages (Figure 2.6a) [62]. White rot fungi wood decay has been used in the pulp and paper industry as a more environmentally friendly method of removing lignin from wood fibers with reduced usage of more harsh chemicals such as concentrated acids and bases [69].

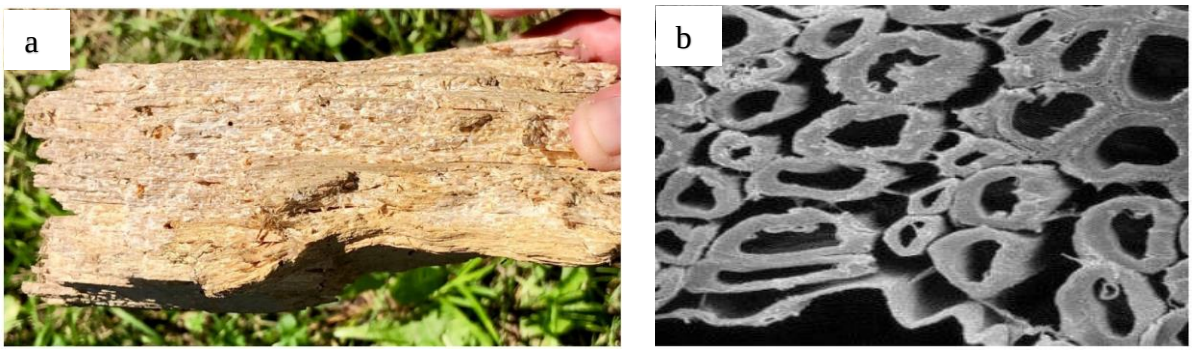

Figure 2. 6**:** a) Photograph of wood residue and b) SEM micrograph of wood after white rot fungi decay in hardwood [62], [68]

2.3 Wood preservation

Wood and wood products are susceptible to deterioration when exposed to conditions that will support the growth and activity of wood-deteriorating organisms such as fungi, insects, termites, and marine borers hence there is the need to protect wood before use[70]. Some wood species are naturally considered durable against wood deteriorating organisms due to the presence of extractives in their heartwood portions whiles many others are considered moderately resistant or susceptible to these agents and hence requires extra protection by way of chemical preservation[2].

Chemical preservatives are applied to wood to extend the lifespan of wood and wood products in service. Additionally, preserving wood prevents the frequent replacement of wood, thus contributing to forest conservation and sustainability. Protected timber has an increased lifespan of 5-10 times the normal and its fire resistance property can be increased 2-3 times to the normal[71]. Some factors to consider before the application of preservatives include the location of the final use of the wood product, conditions of exposure to wood deteriorating agents, and the cost per year of service life of the treated materials [72]. For instance, wood materials that are used in exposed environmental conditions and in contact with the ground including railway ties, poles and posts must be protected to provide appreciable service life [72].

Wood preservative application depends on the wood species, moisture content and the anatomical structure of wood. Heartwood of wood species are resistive to preservative penetration than sapwood. For treated wood to attain a high degree of protection greatly

depends on the preservative used and the penetration level and retention (without leaching) of the chemicals in the treated wood. Other factors may include proper handling of treated wood to prevent cracking and exposing the underlying untreated wood and using the appropriate treated wood for the right application [63], [70]. All these factors and guidelines to achieving effective and quality wood preservation and the recommended end use of the treated material to protect the user are regulated by Federal and state specifications, requirements, and standards of American Wood Protection Association (AWPA) and the building codes in the United States [70], [72]. Different methods have been used to applying chemical preservatives to wood over the years including brush-on and spraying, cold soaking and steeping, thermal process or hot-and-cold bath, Vacuum-pressure methods (full cell process, empty cell processes and modified full cell processes) and vacuum pressure treatment plants [1], [73].

2.4 Current trends in wood preservatives

Wood preservatives are the chemicals substances that are applied to wood to make it resistant by to the attacks of deteriorating agents. The preservatives must be able to protect wood with little or no effect to the end user and the environment. Wood preservatives vary by cost, effectiveness, and fitness for purpose under different conditions. Some major characteristics of wood preservatives includes must be toxic to deteriorating agents with no harm to the wood, high penetrability in wood without leaching, no corrosiveness to metals of contact, odorless, colorless and moisture repellent and most importantly it should be affordable [2]. Conventionally, wood preservatives are classified as organic (oil borne) and water-borne preservatives.

The oil borne preservatives are amongst the oldest preservatives that has been in numerous applications. Notable amongst them for commercial usage in the United States are creosote, pentachlorophenol, copper naphthenate and copper-8-quinolinolate [1]. Heavy oil-borne preservative treated wood are protected from weathering, but the preservatives affect the cleanliness, odor, color, paintability and fire performance of the treated wood [70]. Oil-borne preservatives are mostly used in industrial applications such as utility poles, pilings railroad tiles and other marine and highway constructions.

Water-borne preservatives on the other hand are the most used preservatives in residential and commercial construction applications such as decking and fencing. Chromated copper arsenate (CCA) and ammoniacal copper zinc arsenate (ACZA) were the commonly water-borne preservatives since its inception in the 1950's with the latter mostly used to treat Douglas-fir lumber which CCA does not treat well. Copper azole (CA), alkaline copper quat (ACQ) and micronized copper azole (MCA) were newly introduced water-based preservatives because of the public and regulatory concerns about human exposure to arsenic wood products and arsenic in water streams and hence to serve as alternative non-arsenic contained preservatives [1], [74]. Additionally, some wood decay fungi such as brown rot fungi have been determined to be growing tolerant to copper contained preservatives and hence decay copper contained preservatives treated wood in service [6], [8], [75].

Nowadays, environmentally friendly wood protection is an option of extensive research that covers several different approaches. One of the new trends of preservatives currently being promoted as alternatives to the conventional ones is the use of natural biocides for wood protection. These includes either the use of naturally durable wood species or the use of extracts from plants and animal sources as biocides for preserving wood. For instance, research works have been done using plant extracts such as essential oils[76], tannins[77], and wood extractives [78] for wood protection. Animals sourced extracts includes propolis [79] from honeybees has been extensively studied as a standalone biocide or in combination with other polymers for their antimicrobial activity or against wood-decaying fungi, and mold. Aside propolis as an animal sourced biocide, chitosan[80] has also been extensively studied for its antimicrobial properties against numerous deteriorating organism such as fungi, molds and stains and termites in the realm of wood protection. The use of Chitosan for wood protection is the main focus of this study and hence will be further discussed.

2.5 Chitosan

Chitosan is biopolymer derivative of chitin which the second most abundant polysaccharide to cellulose and is found in the cell walls of fungi and the exoskeleton of crustaceans and insects [81]. It is a polysaccharide comprised of 2-amino-2-deoxy-D-glucopyranose linked together by $\beta(1\rightarrow4)$ linkage and is derived from chitin through the process of alkaline deacetylation (Figure 2.7) [38]. According to Kumirska et al., the source and method of

production of chitin affects the molecular weight, degree of acetylation and other physicochemical properties such as crystallinity of chitosan derived from it [82]. Chitosan is soluble in acidic aqueous solutions and its solubility is highly dependent on degree of acetylation (DA). The degree of polymerization of chitosan which is the chain length of the molecule directly correlates with its molecular weight (MW) and hence the basis for classifying chitosan as a low (50-190 KDa), medium (190-310 KDa), or HMW (310- >375 KDa) molecules [83]. Chitosan contains primary amine groups that makes it suitable for chemical modification through covalent bonding with different functional groups or for crosslinking [84].

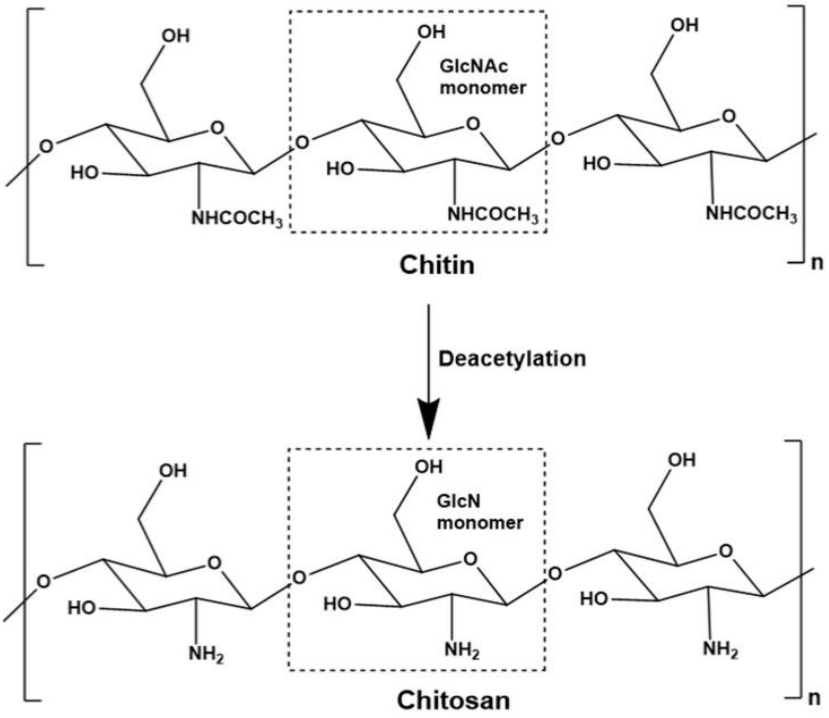

Figure 2. 7**:** Chemical Structure of chitin and its fully deacetylated derivative chitosan [81]

2.5.1 Properties and major applications of chitosan

Chitosan as a semi-crystalline polysaccharide that is soluble in acidic aqueous solutions and hence can combine with other molecules in solution to exhibit improved properties[85]. It is known to be one of the promising and most utilized polymers in current times due to the numerous properties it possesses. Some major biological properties of chitosan include non-toxicity, biodegradability, biocompatibility, and antimicrobial activity against

microorganisms[82], [86]. Additionally, chitosan has exceptional film forming properties with great mechanical strength properties [87]. Due to these numerous properties of chitosan, the polymer has been employed in numerous applications including for drug delivery systems, artificial skin crafting, wound dressing, tissue engineering and bone and cartilage regeneration, cosmetics, photography, water engineering, textiles industry, food and nutrition, packaging industries, agriculture seed treatment, soil amendment and plant disease control, and numerous applications in wood science [82], [88]–[92].

2.6 Applications of chitosan in wood science field

Over the past two decades, chitosan has been extensively studied and employed in numerous applications in the field of wood science. Notable amongst the several investigated applications of the biopolymer includes it use as an adhesive and adhesive systems [93], fire retardants [94], improving mechanical and strength properties treated materials [95], and its extensive usage as a biobased preservative for wood protection against wood decay fungi, molds and wood staining fungi and termites [96]. Detailed discussion on the various studies and applications of chitosan in wood and wood products is further presented.

2.6.1 Adhesive properties of chitosan

The feasibility of using chitosan as a standalone biobased adhesive or employed in adhesive formulation system with other compounds has been broadly studied over the years as an environmentally safe alternative to the conventional unhealthy adhesives. Umemura et al. [97] in one of the earliest studies reported an average dry and wet bond strength of 2.13 MPa and 1.74 MPa respectively for plywood bonded with small solid quantities of chitosan of 16 g/m^3 and 32 g/m^3 spread rates respectively. The improved bond strength recorded were comparable to conventional adhesives such as casein and soybean. Another study investigated the bonding properties of chitosan with varying molecular weight and solid-based spread rates[98]. Results further confirm the good dry bonding of chitosan which increases with increasing solid-based spread rate and to a mild extent increasing molecular weight. However, the wet bond strength was similar for the different chitosan glued samples after immersion in different pH buffer solutions but a sudden loss in wet bond was realised when samples were immersed in 1% acetic acid. Chitosan-glucose adhesive system via

Maillard reaction also revealed an improved dry and wet bonding strength of plywood test samples produced from the adhesive formulation [99]. However, further reports shows that the addition of glucose (10 wt%) to low molecular weight chitosan significantly improved the bond strength even after immersion in 1% acetic acid. An adhesive system comprising of chitosan, glycerol and trisodium citrate dehydrate was also investigated for its bonding properties [100]. It was revealed that 6% chitosan + 5% glycerol + 5 mmol/L trisodium citrate as the best formulation with 6.0 MPa and 1.6 MPa optimum dry and wet bond strengths respectively. The 6% chitosan alone glued test samples recorded a dry bond strength of 6.1 MPa which is comparable to the formulation tested.

Shang et al. [101] studied the adhesive blend of chitosan (C), konjac glucomannan (KGM) and polypeptide for its tensile curing mechanism, physicochemical variations, and changes in thermal behaviour due to the addition of polypeptides. Results from the study shows that the adhesive blend recorded optimal curing temperature of 130 ^{o}C resulting in an improved dry and wet tensile strength properties. An increased storage and loss modulus was also recorded and was attributed to the formation of amides through the reaction of the components of the adhesive blends. Polypeptide addition also improved the wet tensile strength, mechanical properties, and wettability of the adhesive and wood veneer treated [102]. Another adhesive formulation composed of KGM, C and polyvinyl alcohol (PVA) (KCP) recorded a bonding strength that increased with increasing KGM and C. The bond strength of KCP treated plywood after testing was comparable to those of phenol formaldehyde (PF) treated samples [103]. KCP glued oriented cotton stalk board (OCB) revealed mechanical properties that was comparable to urea formaldehyde and PF resins OCB. The improved bond strength reported for KCP formulation was attributed to the presence of strong hydrogen and covalent bonds linking the components of the formulation[104], [105]. Another study reported on medium density fibreboard (MDF) produced with chitosan crosslinked glutaraldehyde (C-Gal) adhesive system and was revealed to have an outstanding bond strength and water resistance which was related to the network formed after crosslinking of C-Gal and the C=N linkages of the self-polymerized glutaraldehyde used [106]. An improved mechanical, and bonding properties was reported for wood fiber composites and MDF produced with C-Gal and lignosulfonate/chitosan-glutaraldehyde adhesive systems respectively that meets requirements of international standards [107], [108]. Ji and Guo [109] also reported an

improved bonding strength and water resistance of the MDF test samples produced with chitosan-lignin adhesive.

Chitosan with different degrees of substitution was employed in a modified adhesive system with alkyl chains and was reported to have an improved water resistance properties compared to native chitosan and maintained sufficient bonding strength after testing treated double-lap wood samples [110]. The chitosan-modified starch adhesive film recorded optimum bonding strength with processing parameters of 145 °C hot-pressing temperature, 183 s hot pressing time and 239 g/m^3 adhesive application to the test samples. The ease to apply and transport with a good shelf life due to low moisture content are some of the characteristic advantages of the chitosan-modified starch film [111].

2.6.2 Mechanical properties of chitosan treated wood

Chitosan treated wood and wood-based products have been investigated for their physical and mechanical properties. Wood Plastic composite (WPC) treated with chitosan and chitosan copper complex (CCC) formulation was investigated for its mechanical properties. Results from the study showed 3% chitosan alone treated WPC recording an average density and tensile strength of 1.15 g/cm^3 and 15.5 MPa respectively whiles CCC treated WPC recorded slightly lower in density and tensile strength properties [112]. High and low molecular chitosan solution stability after repeated impregnation of wood was investigated and its influence on treatment parameters and mechanical properties of the treated wood examined[95]. An average chitosan solution uptake of 15 to16 kg/m^3 was recorded with solution uptake, viscosity, and concentration parameters unchanged. However, an increased pH was observed, whiles the molecular weight decreased which was higher for high molecular weight chitosan. However, mechanical properties were not significantly affected by chitosan impregnation.

Larnøy et al. [113] in another study their study demonstrated the effect of viscosity on chitosan solution uptake in different wood species. It was concluded that the higher the viscosity of chitosan solution, the lower the solution uptake by wood species during treatment. Pre and post treatment factors that influences the fixation of chitosan in wood were evaluated on leached and unleached treated samples [114]. It was proven that pH,

molecular weight, and the types of acid used are the key factors that influences chitosan fixation in wood. Heat modified chitosan treated wood was reported to have resulted in 27% increase in modulus of elasticity and an enhanced hydrophobic nature compared to untreated wood [115]. The high leaching drawback of chitosan from treated wood was further reported in this study [116].

2.6.3 Fire resistance properties of chitosan treated wood

Chitosan biopolymer have also been uses to improve the resistance of treated wood. Heat modified low and high molecular weight chitosan treated wood was investigated for its fire-retardant efficiency (FRE) and results shows an improved FRE for heat modified chitosan treated wood which increases with increasing molecular weight of chitosan[115]. Additionally, a reduction in FRE for heat modified chitosan treated wood compared to chitosan treated samples was observed but compared to untreated wood, a 30-40% higher FRE was revealed. More recently, the fire-retardant activity of quaternized and nonquatarnized nano-chitosan crosslinked with sodium tripolyphosphate (TPP) was investigated [94]. It was reported that nano-chitosan-TPP formulation had significant effect on fire retardant properties which increases with increasing chitosan and TPP concentration when the peak heat release rate and mass loss rate of treated to untreated wood were compared. Zhou and Fu [117] also investigated the flame retardant of chitosan/sodium phytate/TiO_2-ZnO (CH/SP/nano-TiO_2-ZnO) coatings nanoparticle coating film applied on wood samples by layer-by layer assembly. The CH/SP/nano-TiO_2-ZnO coatings was reported to exhibit the best flame retardant performance with approximately six second flame self-extinguishing ability and the coated sample having a limiting oxygen index of 8.4% higher than uncoated wood.

2.6.4 Chitosan for wood protection

2.6.4.1 Antifungal properties of chitosan against wood decay fungi

The past two decades have seen extensive research into the use of chitosan as a biobased preservative against wood decay fungi including white rot, brown rot, soft rots, molds, and staining fungi. Detailed review on the antimicrobial properties of chitosan is presented in the sub sessions below.

2.6.4.2 *In vitro* studies on the antifungal properties of Chitosan against decay fungi

In vitro studies on antimicrobial properties of chitosan were carried out by amending fungi growth media with different concentrations of the preservative and its effects on the growth of the wood decay fungi. Chittenden et al. [118] investigated the growth of sapstain and mold fungi on different molecular weight chitosan amended growth media. It was revealed that chitosan significantly inhibited the growth of the test fungi with low molecular weight being the most effective. Other parameters such as concentration and formulation were also reported to having pronounced effects on the results of the study. Solubilized low molecular weight chitosan amended media was recorded to have reduced the growth of test fungi through fungistatic activity against the staining fungi and two mold fungi *Botrytis cinera* and *Cladosporium herbarum* but not against wood decay fungi. However, chitosan recorded a fungitoxic effect on the stain and mold fungi spores of *Leptographium procerum* and *Trochoderma harzianum* respectively at higher concentrations [119]. Chitosan acetate and chitosan oligomers in amended growth media suppressed the growth of *Leptographium procerum* and *Sphaeropsis sapinea* staining fungi with concentrations of 0.3-0.4% (w/v) but *T. harzianum* mold fungus was not affected. The effect of the chitosan oligomer was determined to be pH dependent with pH of 4 as the most effective [23].

In another study, the growth of *Poria placenta*, *Coriolus versicolor* and *Aspergillus niger* was totally inhibited on chitosan amended media of 1% w/v concentration. However, contrary to results from previous findings, HMW chitosan was reported to be more efficient against mold and staining fungi compared to the LMW chitosan [18]. Eikenes et al. [14] also recorded similar findings but with two brown rot fungi *P. placenta* and *Coniophora puteana* and a white rot fungi *C. versicolor* with 1% HMW chitosan. Furthermore, the growth inhibition was decreased with decreasing molecular weight of chitosan. The changes in the cell wall composition, morphology, and ultrastructure of *T. harzianum* and *Sphaeropsis sapinea* wood fungi due to the effect of chitosan was investigated. [120]. An increased chitin content in the mycelium of *S. sapinea* was reported which was attributed to cell wall deposition mechanism of chitosan leading to changes in cell wall texture and surface morphology after electron microscopy observation.

2.6.4.3 Fungal resistance of chitosan treated wood

Numerous studies on the fungal resistance property of chitosan treated wood have also been reported over the years. The fungal resistance of different sources of chitosan and chitosan-copper sulphate formulation treated maple veneer was investigated in a study by Maoz and Morrell [10]. Chitosan reduced the weight loss caused by the two-brown rot *P. placenta*, *G.t.* and the white rot fungi *T.v.* compared to their control wood veneers which varied from one fungus to another and between chitosan sources. However, the addition of copper had no significant effect on the fungi resistance of the treated wood. Another study investigated the fungal resistance of chitosan copper complex (CCC) treated wood polymer composite (WPC) material [112], [121]. CCC modified WPC recorded weight loss less than 3% which was like those recorded by zinc borated treated WPC after exposure to *G.t.* and *T.v.* decay fungi. 3% CCC was used to treat wood-HDPE composite, and it was confirmed to have improved fungi resistance properties compared to untreated solid wood and untreated wood-HDPE composite especially against the brown rot fungi studied [121]. Alfredsen et al. in their study reported that 5% chitosan treated pine sapwood recorded mass loss of 2.1% after 5 weeks of exposure to *P. placenta* but upon leaching, the mass loss was increased about 10% [18]. Eikenes et al.[14] also recorded similar findings in their study with unleached chitosan treated samples recording lower mass loss than 5% whiles leached samples recorded a pronounced mass loss higher than 5% after exposure to *Poria placenta* and *Coniophora puteana* brown rot fungi. Heat modified 5% chitosan treated samples recorded a slightly higher mass loss compared to unmodified chitosan treated samples for all three fungi of *Poria placenta* and *Coniophora puteana* and *Trametes versicolor* after 8 weeks of exposure [122]. Chitosan treated pine sapwood and beech wood exposed to soft-rot fungi showed an improved dynamic modulus of elasticity (MOE dyn.) compared to untreated samples. Pine treated samples recorded less MOE dyn. loss after 8 months compared to the beech treated wood [25].

2.6.4.4 Termiticidal properties of Chitosan

Chitosan has been determined to exhibit antimicrobial properties against termites. The efficacy of chitosan treated wood against *Reticulitermes flavipes* and *Reticulitermes virginicus* termites was investigated by Raji et al. [30], [31]. Termite mortality was increased with increasing concentration of chitosan for *R. flavipes* with more than 94% mortality after exposure to chitosan treatments more than 2%. For *R. virginicus*, a 100% mortality was

reported for all chitosan treated wood exposed to it [30]. Mass loss in chitosan treated wood also decreased with increasing concentration of chitosan. Chitosan treated wood exposed to *R. flavipes* termites was examined for effect on termites' guts bacteria [32]. Results shows two gut bacteria phyla of *Firmicutes* and *Actinobacteria* were significantly affected by chitosan with the former having lower abundance with chitosan treatment whiles the latter was lower in unexposed and starved termites indicating starving of termites can also significantly influence gut bacteria.

2.6.5 Mode of action of antifungal properties of chitosan

The antimicrobial properties of chitosan has been reported in several studies especially against fungi species [123]–[125]. However, the exact mechanism of how chitosan exerts its antimicrobial activity is yet to be fully understood. Therefore, two main hypotheses have been widely known to explain this phenomenon. The first and the most widely accepted hypothesis states that chitosan is cationic in nature and gradually conveys its positive charge from the NH_3^+ groups of glucosamine which interacts with the negatively charged cell surfaces of the fungi causing leakage of intercellular substances that either inhibits their growth or in extreme cases the death of the of the fungi (Figure 2.8). The other assumes that the water-binding ability, metal chelating properties and the ability of chitosan to interact with the DNA of fungi may better explain the antifungal mode of action of chitosan [126]–[132]. Key factors that influence the antimicrobial actions of chitosan includes the molecular weight, degree of acetylation, degree of polymerization and pH with molecular weight having the greatest effect [133], [134].

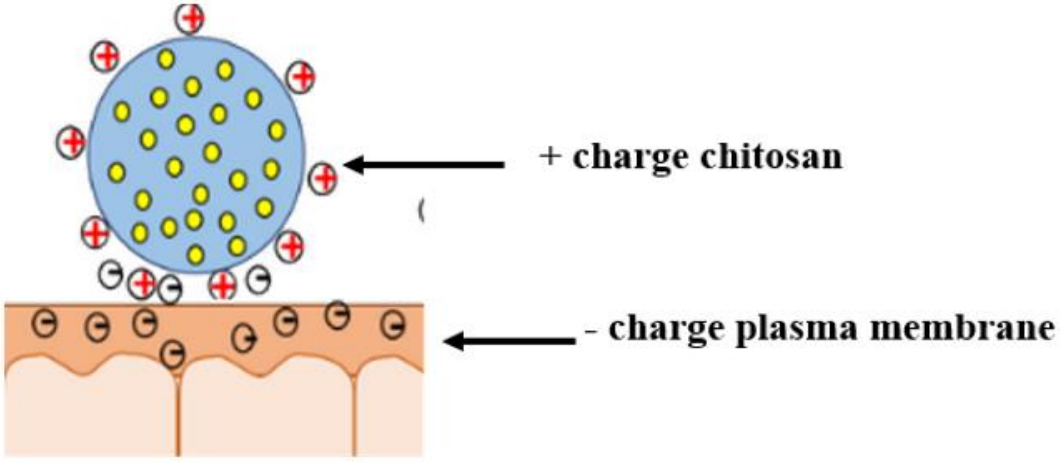

Figure 2. 8**:** Schematic depiction of the mode of action of the antifungal properties of chitosan

2.7 Genipin

Genipin is a biobased crosslinking compound that has been extensively studied for its properties. It is obtained from geniposide that is an isolate from the fruits of *Gardenia jasminoides* and *Genipa americana*[135], [136]. It is made up of the molecular structure $C_{11}H_{14}O_5$ which is iridoid glycoside, known to be one of the main ingredients in the fruits of the species [135], [137] (Figure 2.9). It is white crystalline, biocompatible, and biodegradable water-soluble powder that has a bifunctional crosslinking property[136]. It reacts and crosslinks with biopolymers containing amine groups such as collagen, gelatin and chitosan[137], [138]. Genipin has low toxicity levels and was determined to be 5000 to 10,000 times lower in toxicity than glutaraldehyde which is another cross-linking agent [139], [140].

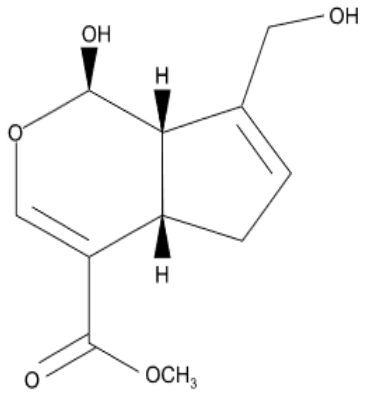

Figure 2. 9**:** Structure of Genipin[141].

2.7.1 Genipin crosslinked chitosan and its applications

Genipin reacts with a chitosan which is an amino group containing polymer to produce a formulation with a dark blue color change (Figure 2.10) [142], [143]. This reaction occurs as there is a nucleophilic on genipin specifically the olefinic carbon atom at the C3 position is attacked by the amine groups of chitosan leading to the opening of the dihydropyran ring under acidic or neutral conditions [135]. This is followed by a much slower reaction leading to the formation of amides through the reaction of the amino group on chitosan with the ester group (by C-11) of genipin [144]. According to Muzzarelli et al. a concurrent polymerization reaction could also take place between the already linked molecules of genipin and the amino groups of chitosan by short genipin copolymers. [90]. There is the occurrence of an additional condensation reaction which makes a genipin crosslinked products more stable than those of glutaraldehyde [135]. Some major uses of genipin crosslinked chitosan includes antimicrobial activity against microorganisms [145]–[148], for drug delivery and release systems [36], [137], [139], [142], [144], [149]–[151], bone and tissue regeneration engineering [135], [141], [145], [152]–[155], and as a cellular adhesive agent [138].

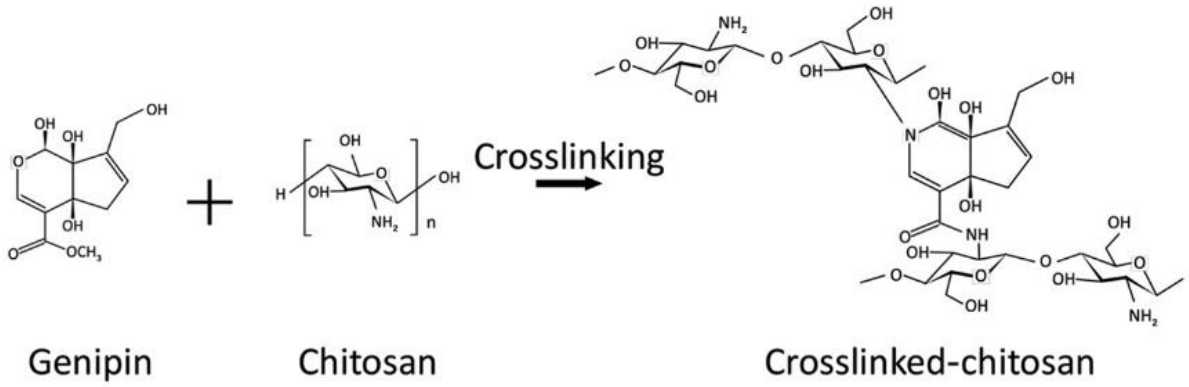

Figure 2. 10**:** Reaction equation of genipin reacting with chitosan [135]

Chapter 3: Materials and Methods

This chapter clearly elaborate on the materials and testing procedures followed in acquiring all available data used in this study.

3.1 Materials

Low molecular weight chitosan (50 -190 kDa) with a deacetylation degree of 75%, genipin (≥ 98 % purity) crosslinking agent, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and Calcofluor White stain were purchased from MilliporeSigma (Burlington, MA, USA). Potassium hydroxide (KOH) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Other chemicals such as acetic acid and ethanol used were of analytical grade of reputed companies. Two brown-rot fungi: *G.t.* and *R.p.* and two white-rot fungi: *T.v.* and *I.l.* purchased from ATTC were used for the decay test.

3.2 Methods

3.2.1 Preparation of genipin-crosslinked chitosan precursor solutions

Chitosan (C) solutions of four different concentration levels, including 0.5, 1, 2 and 3%, were prepared by dissolving the corresponding amount (g) of chitosan powder in 1% v/v acetic acid. The mixture was then vigorously stirred under an overhead mechanical stirrer (JJ-1 Precise Strength Power mixer) for 4 h at room conditions until complete dissolution of chitosan solutes (Figure 3.1a). Genipin solution was prepared by fully dissolving 5 mg of genipin in 1 mL ethanol. The genipin solution was added to the chitosan prepared to solutions and was vortex mixed for 5 min to obtain the genipin-crosslinked chitosan (GC) precursor solutions of four different concentrations for further use. The addition of genipin was based on 0.05% wt. amount of chitosan in each concentration of the chitosan prepared solutions. A successful crosslinking between genipin and chitosan results in a bluish-black color change of the final GC solution (Figure 3.1b).

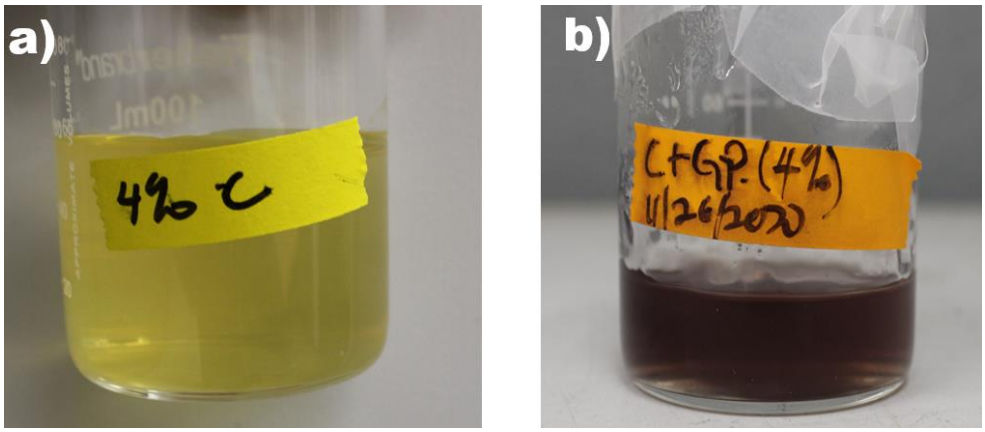

Figure 3. 1**:** a) Chitosan solution and b) Genipin crosslinked chitosan solution

3.2.2 Casting of genipin crosslinked chitosan films

A portion of the prepared solutions of 3% C and GC were casted into films following the methods described by Eulálio et al. [156]. Briefly, the prepared solutions of C and GC were poured into a 90 mm diameter glass petri dish and spread to a uniform thickness and oven dried at 60 °C for 48 h. The dried films were carefully peeled out from the dish, freeze dried and used for further analysis.

3.2.3 Characterization of genipin-crosslinked chitosan films

Qualitative characterization of the C and GC casted films was done by Fourier Transform Infrared (FTIR) Spectroscopy methods whiles the thermal stability and degradation of GC films was conducted using the thermogravimetric analysis (TGA).

3.2.3.1 Fourier transform infrared (FTIR) spectroscopy

FTIR is used to study the chemical changes that occurred after crosslinking genipin with chitosan. The spectra's (triplicates) of C and GC cast film samples were obtained using a Nicolet - iS10 FTIR spectrometer (Thermoscientifc, Madison, WI, USA) from 500 to 4,000 cm−1 with 64 scans, on a smart orbit diamond crystal. The spectra were averaged, and baseline corrected using the OMNIC v9.8 software.

3.2.3.2 Thermogravimetric analysis (TGA)

TGA is used to study the thermal decomposition and stability of C and GC film samples. The TGA was performed on ~5 mg film samples using the Perkin-Elmer TGA- 7 instrument. The

samples were cut into pieces and heated from 30 to 800 °C at a heating rate of 20 °C/min under a nitrogen flow of 30 mL/min. The actual stages of degradation and the maximum degradation temperature of the samples were determined with the aid of the derivative thermogravimetric (DTG) curves.

3.2.4 *In vitro* antifungal properties of genipin-crosslinked chitosan

The *in vitro* antifungal properties of GC against four wood-decaying fungi were first screened using malt agar media as substrate. Specifically, the control substrate was prepared by dissolving 2% malt extract, 1.2% agar, 0.2% yeast in distilled water and was sterilized using the autoclave. C or GC amended plates were obtained by mixing different amounts of 4% C and GC stock solutions with the sterilized malt-agar solution to produce 0.1, 0.5 and 1% (v/v) treatment solutions, of which 12.5 ml were measured and were cast into a 90 mm diameter petri dish (or plate). The solidified plate was then inoculated with a uniform mycelium cut (~10 mm^2) from the edge of an actively growing colony. All inoculated plates were incubated at 75% relative humidity and 25°C temperature in dark in an environment chamber for 14 d. Five replicate plates were prepared for each treatment concentration. Where the test fungi failed to grow on amended growth media after the test period, the original plugs of inoculum were transferred onto fresh malt agar growth media and incubated in the controlled environment chamber to determine whether the inhibitory effect of either C or GC was fungistatic or fungi-toxic [19].

The plates were monitored by capturing daily pictures of the inoculated plates in a custom-made box for 14 d (Figure 3.1). The growth area of each plate was calculated by ImageJ software [157] using the following the equation below:

$$\text{Fungal growth rate (\%)} = \left(\frac{A_1 - A_0}{A_2}\right) \times 100$$

Where: A_0, A_1 and A_2 are the measured areas of initial fungal inoculate, area of fungi growth for each day and area of the petri dish used for the study, respectively.

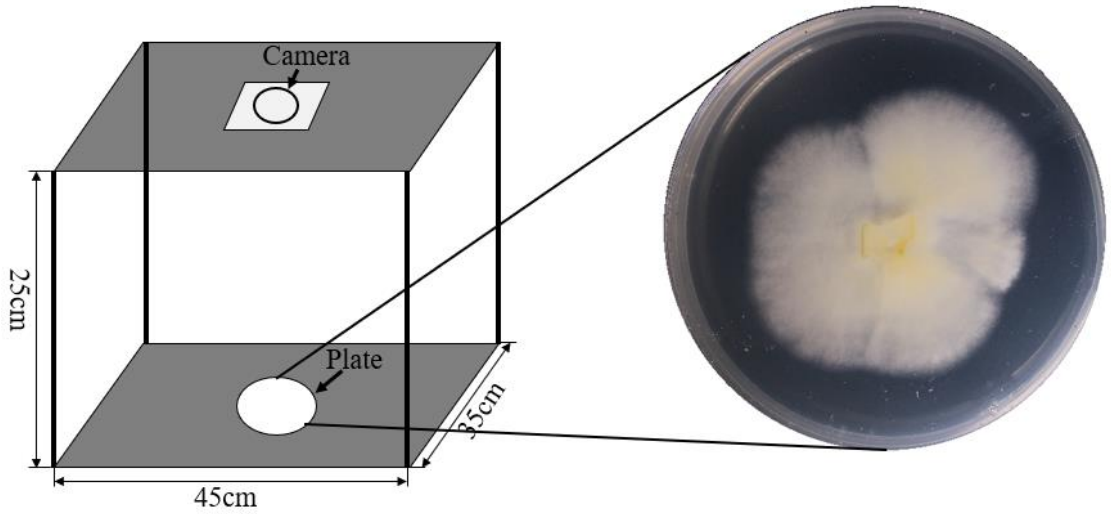

Figure 3.2**:** Schematic diagram of the custom-made soft box for daily picture taking of fungi plates

3.2.5 Light and fluorescence microscopic observation of fungal

The fungal microscope slides were prepared per the description methods by [158] with minor modifications. First, either the control or 0.5% amended malt agar media cuts (~10 mm^2) was transferred onto a sterilized base slide, followed by inoculating with actively growing fungal tips of the testing fungus. The 0.5% treatment concentration was chosen because it retarded the growth of all fungi to some extent but allowed for some growth on the amended media, which makes it possible to study the mycelia morphological changes. A cover slide was placed on top of the inoculated media and the whole set was transferred into a plastic petri dish containing a moist sterile filter paper. The plates were incubated at 75% relative humidity and 25°C in a controlled environment chamber in the dark until adequate growth was attained. The top slide covered with mycelium was then separated and mounted on a new base slide with drops of sterile DI water for light microscopy observation using the Olympus BX51 microscope with a DO 70 camera.

For blue-light fluorescence microscopy observation of nuclei changes [159] the fungi mycelia on the slides were stained by first adding in drops of 10 µg/ml DAPI solution, followed by 10% KOH solution and finally Calcofluor white stain. Five replicate slides were prepared for either light or blue-light fluorescence microscopy observation and at least 20 micrographs were observed for each treatment.

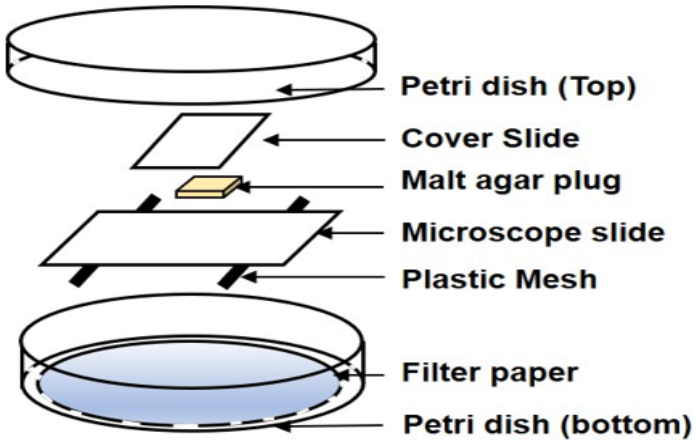

Figure 3. 3**:** Schematic diagram of how the microscopic slides were prepared for the analysis [160]

3.2.6 Wood samples preparation

Sapwood without visible defects from southern pine (*Pinus taeda*) and poplar (*Populus alba*) were cut into dimensions 5 mm × 18 mm × 18 mm (T × L × W, end-matched) according to the requirements of AWPA E22-16 standard [161]. The excess splinters on the edges of the cut samples were removed using a fine grit sanding paper. A total of 480 wood samples, half for southern pine and another half for poplar, were used for the study.

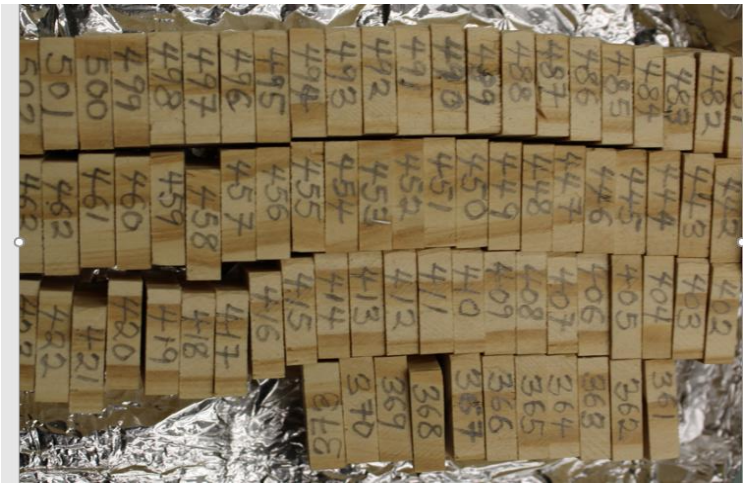

Figure 3. 4**:** Prepared wood samples

3.2.7 Preservatives treatment by impregnation of wood in genipin-crosslinked chitosan precursor solutions

Prior to treatment procedures, the wood samples were oven-dried at 60°C for 48 h and weighed (nearest 0.0001g). The wafers were then vacuum impregnated (86 kPa) with C and GC precursor solutions at concentrations of 0.5, 1, 2 and 3% (v/v) for 60 min following the methods of the AWPA E10 standard [162] (Figure 3.5). Upon impregnation, the samples were allowed to equilibrate in solutions for approximately 24 h, after which the excess treatment solutions on the surface of the samples were wiped off and the wet weight recorded. The treated wafers were then oven-dried at 60 °C for 48 h to obtain the final weight. Wood samples treated with distilled water and 1% AcOH were used as control.

Figure 3. 5: Impregnation of wood with GC precursor solution

3.2.8 Quantification of genipin-crosslinked chitosan in wood by retention and mass gain

The amount of the impregnated preservatives retained in wood samples was measured in terms of retention and mass gain following AWPA E10 standard [162]. Retention after treatment was calculated as follows:

$$\text{Retention (kg/m}^3\text{)} = \frac{GC}{V} \text{ x } 10$$

Where: G= ($m_{trt} - m_{untrt.}$) = grams of treating solution absorbed by the wood blocks (initial weight of block before treatment subtracted from the initial weight plus the treating solution absorbed); C = grams of preservative in 100 g of treating solution; and V = volume of block in cubic centimeters.

The percentage mass gain of the wood wafers after treatment was determined as follows:

$$\text{Mass gain (\%)} = \frac{(m_{trt.} - m_{untrt.})}{m_{trt.}} \text{ x } 100$$

Where $m_{untrt.}$ and $m_{trt.}$ are oven-dried mass of samples before and after treatment, respectively.

3.2.9 Leaching test on GC treated wood

Leaching test was conducted on half of the wood wafers according to the procedures of Standard AWPA E11[163]. In short, 12 replicates of wafers from each treatment were first submerged in 78 ml of deionized water (wood: water volume ratio at 1:4) in separate beakers for 1 hour and the leachate was removed and replaced with fresh DI water. Then the beakers containing wood samples were shaken at 100 rpm for 6 h at room conditions and the leachates were removed again by replenishing with 78 ml DI water. This step was repeated at 24, 48 and thereafter 48 h until a total of 9 leachates were collected for a total period of 14 days. The wet mass and the oven-dried (60^{o}C for 48 h) mass of the test samples at the end of leaching test were recorded to calculate the mass gain and retention after leaching using the equations above.

3.2.10 Fungal resistance of treated wood

The effect of chitosan-genipin formulations on fungal resistance of both southern pine and poplar was evaluated per standard AWPA E22-16 [164] with a modification on sample sterilization. Specifically, the samples were sterilized by spraying with 70% ethanol solution on their surfaces and drying in a biosafety hood for 1 h. This process was repeated for three times. Six replicates from each treatment group were exposed to the test fungi in soil culture bottles and the culture bottles were incubated for 4 weeks at 25 °C and 75% RH in the dark. Both leached and un-leached samples were used for the soil block test. At the end of

exposure period, the mycelia from the decayed wood were carefully brushed off, and the wet mass and the oven-dried mass (60 ºC for 48 h) were recorded. The moisture content and mass loss of decayed wood were calculated as follows:

$$\text{Moisture content (\%)} = \frac{(m_{wet} - m_{expo.})}{m_{expo}} \text{ x } 100$$

$$\text{Mass loss (\%)} = \frac{(m - m_{expo.})}{m} \text{ x } 100$$

Where m and $m_{expo.}$ are the oven-dried mass of wood wafers before and after fungal exposure, respectively. $m_{expo.}$ represents wet mass of the wood samples after decay.

3.2.11 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze all the data using the commercial analytical software, SAS (9.4, SAS Institute Inc., Cary, NC). Results from the analysis were interpreted at a 5% significance level.

Chapter 4: Results and Discussion

4.1 Characterization of genipin-crosslinked chitosan

4.1.1 Fourier transform infrared (FTIR) spectroscopy

The chemical changes due to crosslinking of chitosan with genipin were investigated by FTIR spectroscopy, as shown in Figure 4.1. Chitosan sample showed characteristic bands at 3257 cm^{-1}, 1631 cm^{-1}, 1540 cm^{-1} and 1375 cm^{-1} which are corresponding to the stretching vibrations of -OH, N-H, amide group I (C=O), amide group II (NH_2) and -C-O-H of the primary alcoholic group, respectively [36], [136], [139]. Upon crosslinking with genipin, the bands at 1540 cm^{-1} and 1631 cm^{-1} in the spectrum of C sample shifted to 1543 cm^{-1} and 1639 cm^{-1}, respectively, due to the nucleophilic substitution of the ester group on genipin by the primary amine group on chitosan (Figure 1C) [38]. Additionally, the increased band intensity at 1708 cm^{-1} (C=O stretching) and the changed ratio of the two bands at 1375 cm^{-1} (C-O-H of the primary alcoholic group) and 1402 cm^{-1} (C-N stretching vibration) were observed in GC [36], as compared to those bands seen in C. These changes confirm the formation of GC, resulting in heterocyclic amines under acidic conditions [149].

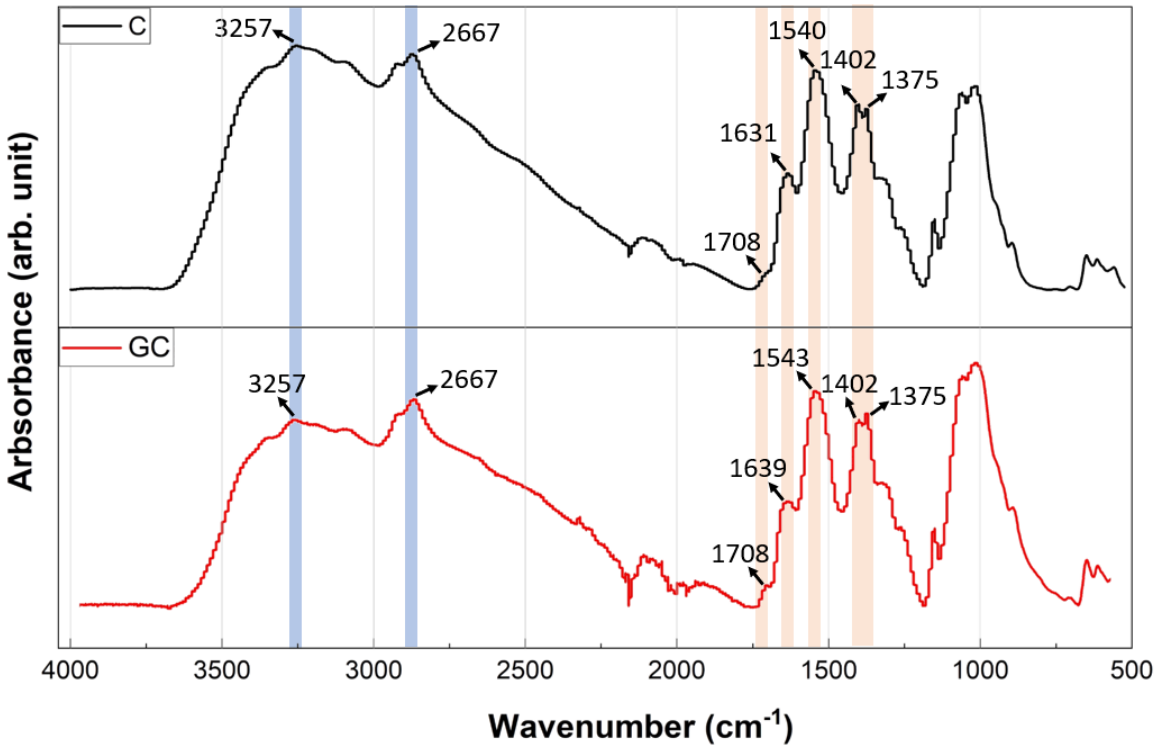

Figure 4. 1**:** FTIR Spectra of C and GC films

4.1.2 Thermogravimetric analysis (TGA)

The formation of GC was further confirmed by studying the thermal stability differences between C and GC (Figure 4.2). Both samples show a mass loss before 150 ^{o}C due to water evaporation. However, the mass loss of GC in this region is much higher than C sample, indicating the increased water content of GC [36]. However, GC sample shows higher thermal stability from 150 to 300 ^{o}C than that of C, which was attributed to the dehydration of the saccharide rings, depolymerization, and decomposition of the polysaccharide structure [36]. The onset decomposition temperature and maximum decomposition temperature of GC were 268^{o}C and 299^{o}C, respectively, which were slightly higher than that of C sample at 265^{o}C and 291^{o}C, indicating crosslinking chitosan with genipin stabilized the structure of chitosan polymer, thus the increased thermal stability of GC [149], [165], [166].

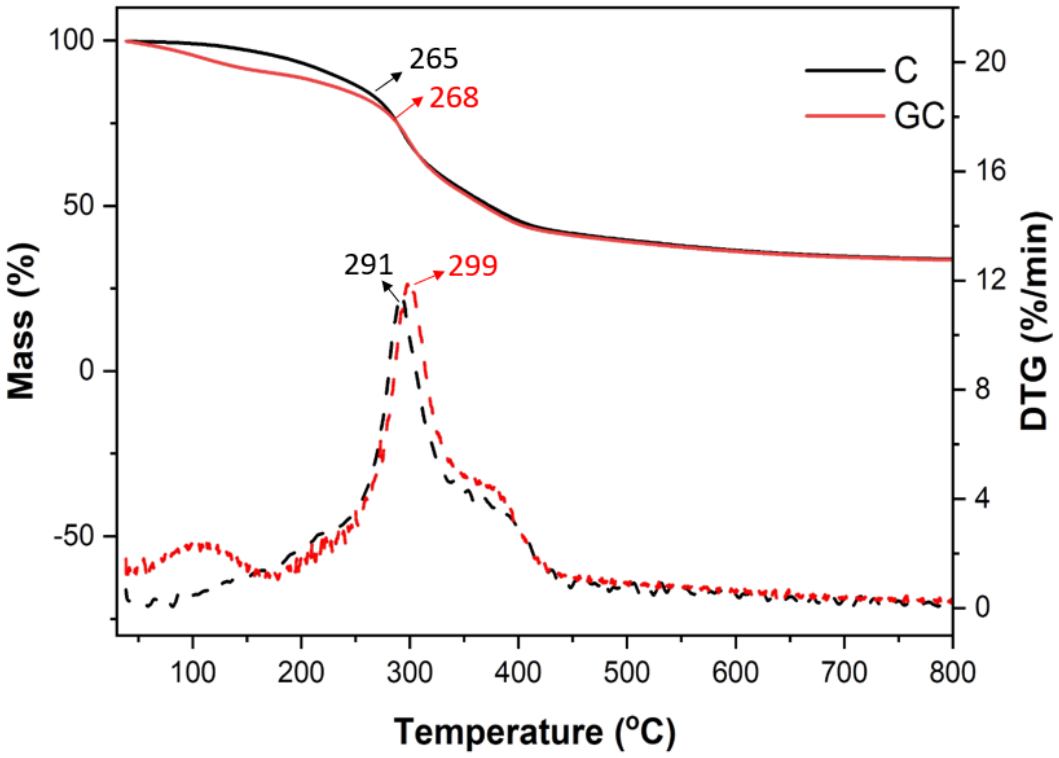

Figure 4. 2**:** TGA and DTG thermograms of C and GC films

4.2 *In vitro* antifungal properties of genipin-crosslinked chitosan

The average growth rate of four common wood decay fungi on C and GC amended plates as well as the unamended plates over the 14-day incubation period is shown in Figure 4.3. Overall, although different fungi responded differently to various concentrations of the testing solutions [7], [167], the growth of all these fungi was significantly inhibited as a function of the concentration of both C and GC, as compared to the growth rates on control plates (Figure 4.3). Specifically, *G.t.* is the most sensitive to C and GC treatment among the four tested fungi with less than 3.5% growth rate being recorded at C and GC concentrations of 0.5% or higher (Figure 4.3 a). More importantly, even though the growth rates of *G.t.* on GC amended plates were lower than those in C amended plates, the differences were not statistically significant ($p > 0.05$).

Nevertheless, unlike *G.t.* fungus, *R.p.* was the least sensitive to C and GC concentration below 0.5% and its growth was completely inhibited at C and GC concentration of 1% throughout the 14 d of the study (Figure 4.3b). To further determine the minimum inhibition

growth concentration for *R.p.*, the original mycelium plugs on the 1% C and 1% GC amended plates of *R.p.* were transferred onto a fresh malt extract agar and the plates were incubated in the environmental chamber. It was observed that *R.p.* regrew quickly and fully covered the plates on day 7, indicating that both C and GC are fungistatic rather than fungitoxic against *R.p.* at 1% treatment level [119]. In terms of *T.v.* and *I.l.*, both fungi responded to C and GC similarly with *I.l.* (Figure 4.3d) being more sensitive to the treatment effects than *T.v.* at C and GC concentration of 0.5% or higher (Figure 4.3c) [18]. However, when comparing to brown rot fungi tested (*G.t.* and *R.p.*,), these two white-rot fungi were less sensitive to C and GC solutions at 1% concentration.

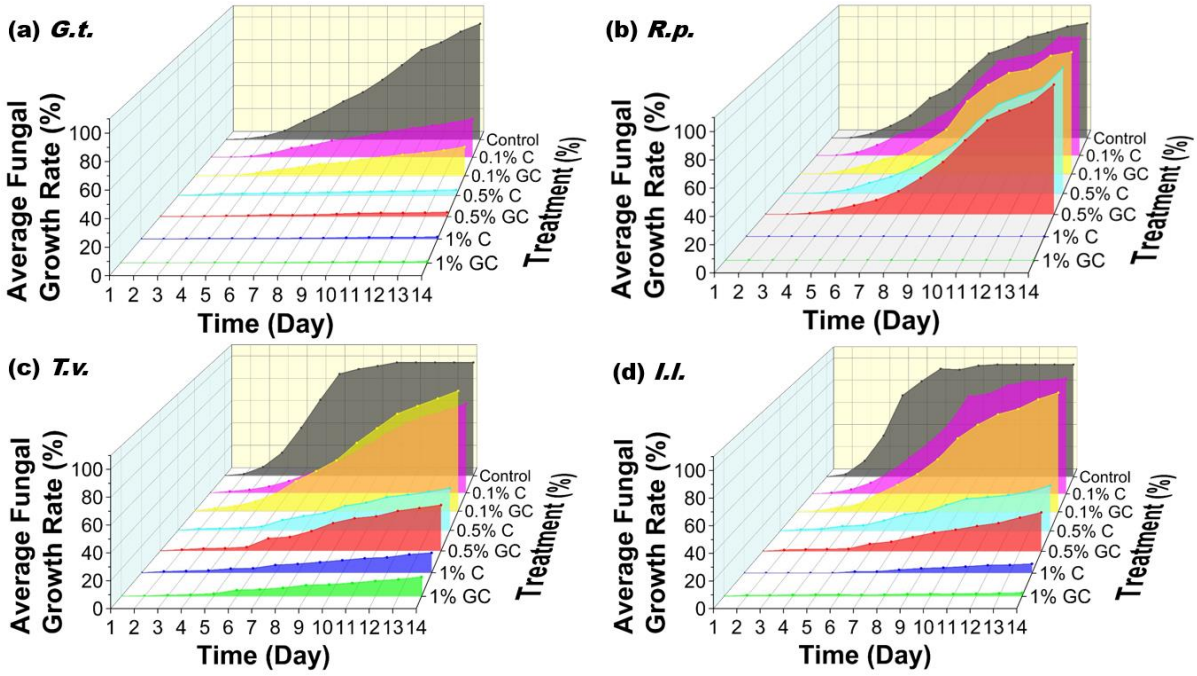

Figure 4. 3**:** Average growth rate of four common wood-decaying fungi exposed to different treatment levels of chitosan (C) and genipin-crosslinked chitosan (GC) amended malt agar substrates over a 14-day incubation period

Figure 4.4 shows photos of plates for all four fungi due to the effects of C and GC on the 14th day of incubation. As stated previously, the growth of fungi decreased with increasing treatment concentrations for all fungi tested except for *R.p.*, which completely covered the plates on both 0.1% and 0.5% C and GC amended plates on day 14. The preservatives treatments also caused browning of the mycelium of all the fungi tested except for *R.p.*. For

example, a deep brown color change of mycelia was observed in both C and GC amended plates of *G.t.* while a light brown coloration was observed in *T.v.* and *I.l.*. The intense coloration of mycelium is likely attributed to the response of fungi to stress induced by the presence of chitosan or genipin cross-linked chitosan in the amended media [118].

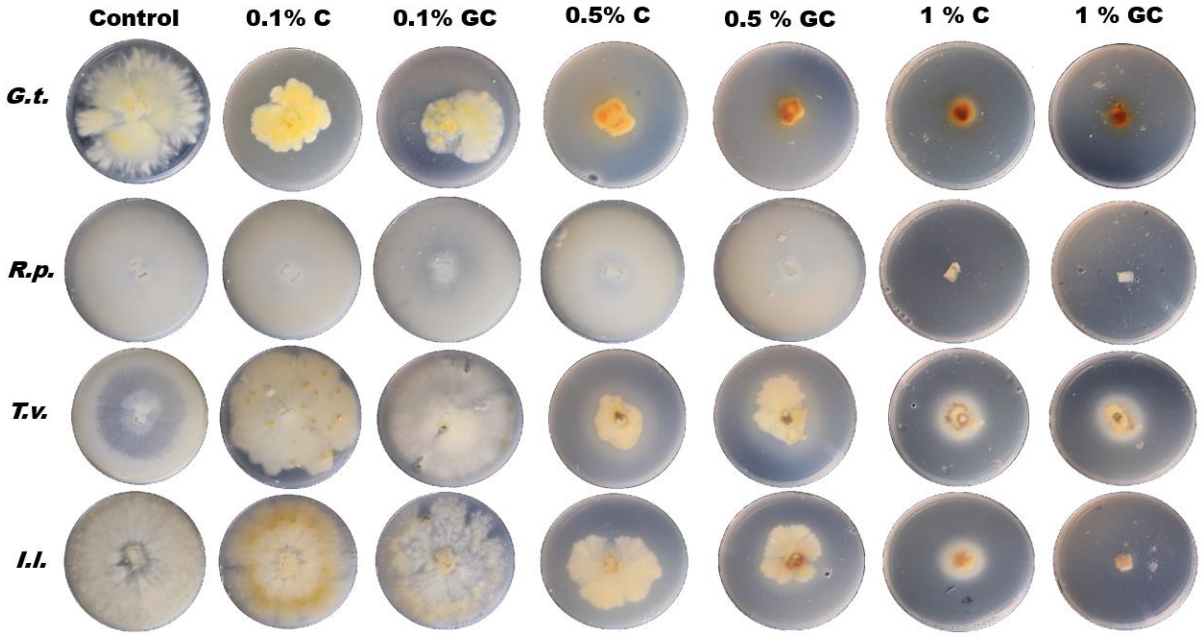

Figure 4. 4**:** Photographs of the four common wood-decaying fungi on amended and unamended plates on day 14th

4.3 Light and fluorescence microscopic observation of fungal growth

Morphology changes of the four wood-decaying fungi due to C and GC treatments were observed under bright field and fluorescence microscopy, as shown in Figure 4.5. From the light transmission micrographs (Figure 4.5a), the terminal hyphae of *G.t.* from both C and GC amended plates showed segmentation along the septum with cytoplasm aggregation and excessive branching compared to control. In the case of *R.p.*, *T.v.* and *I.l.*, distortion of fungal hyphae and precipitation of cytoplasm were observed in all the treated groups. The results conform with a previous study stating that chitosan affected the morphology of the root fungi by altering plasma membrane, thickening cell wall, causing hyphal distortion and cytoplasm aggregation [167].

In addition to the morphology changes observed in brightfield, the blue-light fluorescence micrographs (Figure 4.5b) provide more information regarding the changes of nuclei due to

effect of C and GC. In control hyphae, the observed nuclei were intact in all the four fungi and these nuclei were more uniformly distributed in white-rot fungi, *T.v.* and *I.l*, than those of in the brown rot fungi, *G.t.* and *R.p.*. In contrast, the nuclei were either split or melted upon preservative treatments [167]. The significant changes in fungi morphology are possibly related to the penetration of chitosan-based preservatives on the plasma membrane where the amino groups bind with the negatively charged phospholipids through electrostatic or ternary interactions, thus altering the membrane fluidity, and leading to changes in the fungal cell [168].

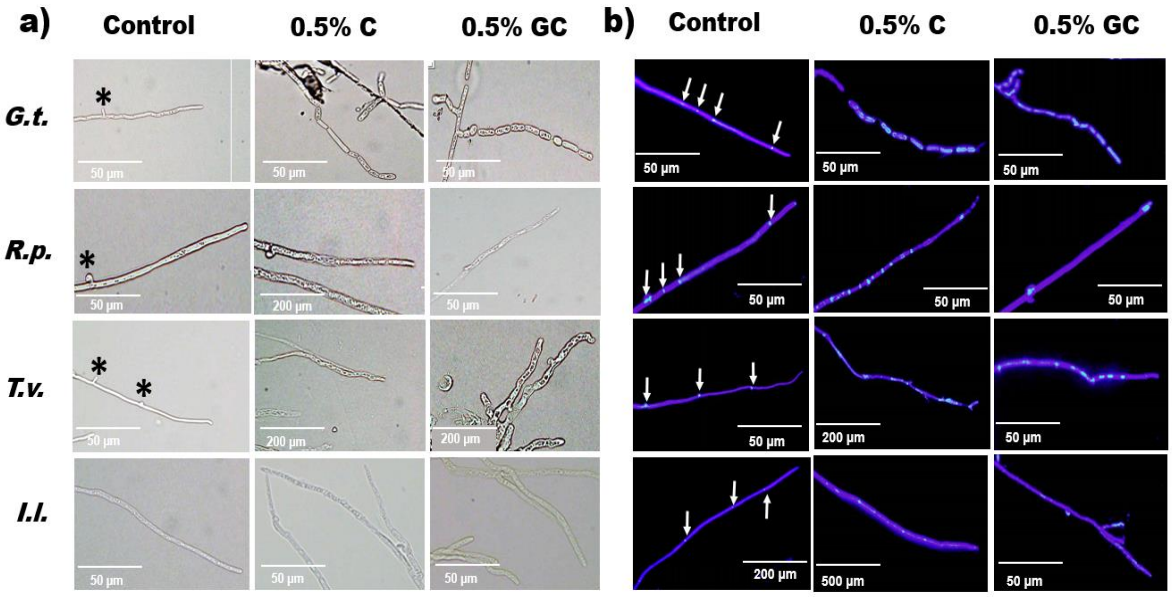

Figure 4. 5**:** Micrographs of a) light transmission and b) blue-light fluorescence study showing morphological changes in the hyphae strands and nuclei of all four decay fungi species due to the effect of chitosan and genipin cross-linked chitosan formulation. The stars (*) in a) indicate the clamp connections are widely observed in control of all the tested fungi with more disperse distribution in *I.l., thus not showing in the picture at current magnification* while the arrows (↑) in b) show the intact nuclei seen in the fungal cells

4.4 Quantification of genipin-crosslinked chitosan in wood by retention and mass gain

Retention and mass gain are two of the major indicators that determine how effective the C and GC preservatives penetrated and are retained in wood after treatment. This section also presents the retention and mass gain of C and GC wood after leaching test.

4.4.1 Retention of C and GC treated wood

The retention of softwood and hardwood after vacuum impregnation (Figure 4.6a) and after leaching test (Figure 4.6b) are presented in Figure 4.6. Generally, the retention for both softwood and hardwood samples increase with increasing concentration (Figure 4.6a) regardless of the leaching test. In particular, retention of hardwood treated samples was significantly higher ($p<0.05$) than those of softwood. At treating concentrations of 0.5%, 1% and 2%, the retentions of GC-treated samples were not significantly different from those of C-treated samples ($p>0.05$). However, 3% GC treated samples recorded a significantly higher ($p<0.05$) retention compared to 3% C treated samples for softwood while opposite observations were found for hardwood.

A similar retention results were recorded for all the tested wood samples after leaching test except that a significant increase in retention was observed for 3% C and GC samples (Figure 4.6b). This may be attributed to the entrapment of the water in wood cells by C and GC polymeric membrane that were retained in wood after leaching [169], [170].

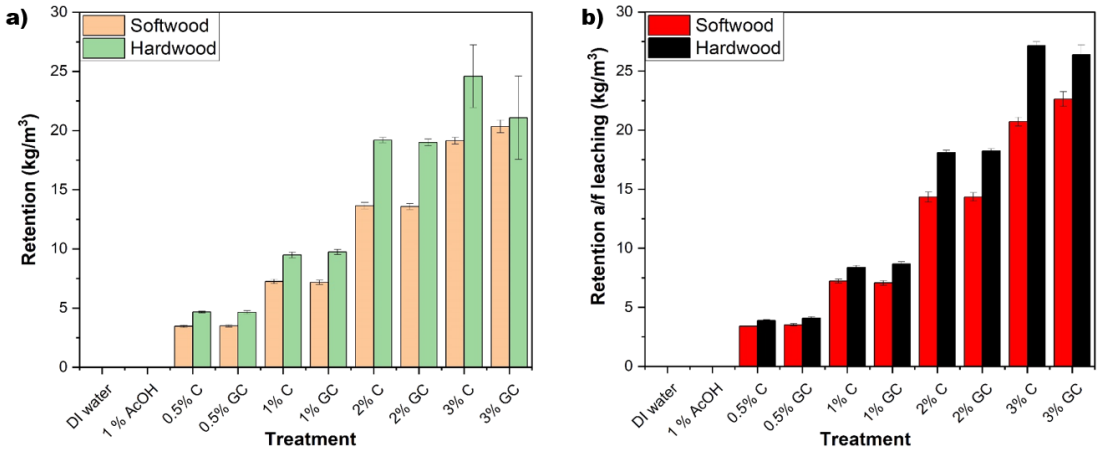

Figure 4. 6**:** Retention of C and GC preservative-treated wood before and after leaching test

4.4.2 Mass gain of C and GC treated wood

For mass gain, water and 1% AcOH treated samples had negative values (less than 1%) with water treated samples recording a significantly lower ($p<0.05$) mass gain than 1% AcOH treated samples for both softwood and hardwood (Figure 4.7a). The negative mass gain values are due to the leaching of the extractives from the wood [171]. There was a similar increase in mass gain which increases with the increasing concentrations of the treating solutions for both C and GC treatments in the two wood species [22].

However, mass gains after the leaching test were significantly decreased across all the treatments (Figure 4.7b). Especially at C and GC concentrations below 1%, all the treating chemicals were leached out. Nevertheless, approximately 0.8% and 1.5% of C or GC are retained in the wood samples after leaching. However, for 2% C and GC treated samples there is no significant difference ($p>0.05$) in mass gain after leaching except for the 2% GC treated hardwood samples. In the case of 3% C and GC treated samples after leaching, softwood from both treatment records significantly higher mass gain ($p<0.05$) than the hardwood. These results show that the amount of the C and GC retained in wood due to leaching increases with increasing treating concentration and varies by wood species. Overall, the results from the leaching test show that crosslinking genipin with chitosan did not reduce the leaching rate of C in treated wood.

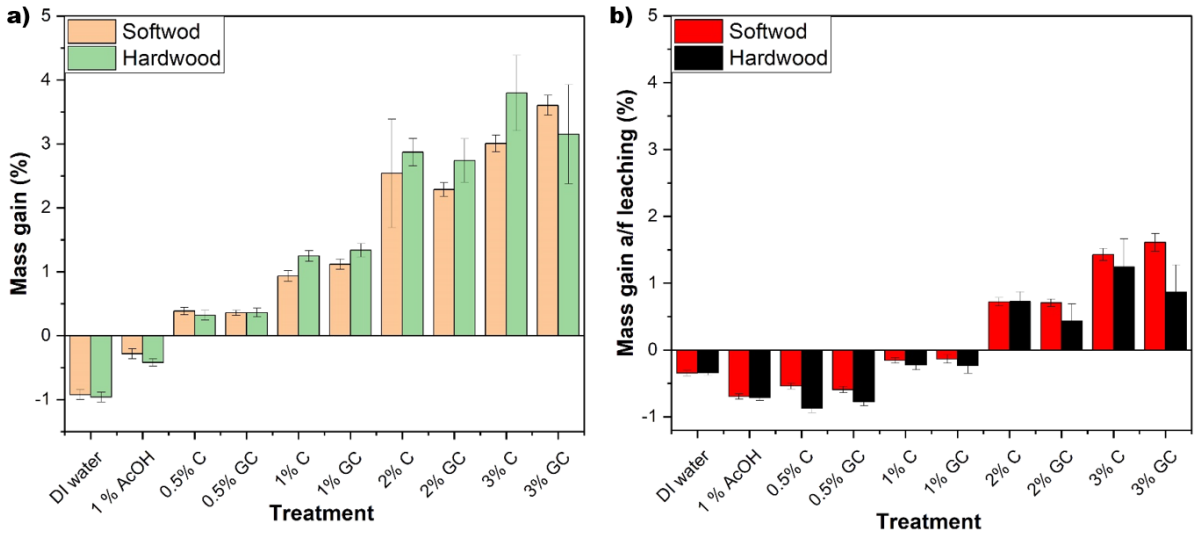

Figure 4. 7**:** Mass gain of C and GC preservative-treated wood before and after leaching test

4.5 Decay resistance of GC against wood-decaying fungi

The mass loss due to 4-week of brown rot and white rot decay are shown in Figure 4.8. In general, the controls groups, including water and 1% AcOH treated wood samples, showed average mass losses of more than 20% and the moisture content of all the decayed samples were between 36% and 65%, indicating the test was valid. Specifically, for *G.t.*, C or GC treated samples generally have a significantly lower mass loss than those of the control groups regardless of leaching test except for 1% AcOH w/o leaching, 0.5% GC, 1% C and 1% GC w/ leaching tests ($p<0.05$). In comparison to C treated groups w/ leaching, GC samples w/ leaching have a significant higher mass loss when the treating concentrations were below 2%. In terms of brown rot fungus *R.p.*, it is more aggressive on C or GC treated wood than *G.t.* (mass loss of ~40% VS ~20%) and the overall mass losses at 3% treatment levels are significantly lower than those of control and lower concentration treatments.

Similar results were obtained from white rot decay fungi, *T.v.* and *I.l.*. Overall, the mass loss of chitosan-based preservatives treated wood due to decay in this study is higher than previously reported [14], [18]. Also, comparing our current results to AWPA listed and commercially utilized copper-based preservatives treated wood samples (including CCA, CA and ACQ), unleached C and GC treated samples recorded about 10% high mass loss to unleached commercial preservative treated wood samples with mass losses generally less than 3% after exposure to both brown and white rot fungi [172], [173]. It is however worth noting that there are some conflicting results regarding the mass loss of chitosan treated wood against wood rotting fungi. For example, in a study reported by [18] mass losses of 16.7%, 2.3% and 2.1% were observed in unleached C treated samples at a concentration of 1%, 2.5% and 5%, respectively, when exposed to *P. placenta*. Another study finds that when treating wood with 2.5% and 5% commercially produced chitosan solutions (with different molecular weight), mass losses due to decay of *P. placenta* and *T.v.* are less than 5% [14], [115]. However, it was also reported that unleached 5% chitosan treated samples had a mass loss of approximately 18% after exposure to *T.v.* [174]. The differences in the durability testing results could be resulting from the various sources of chitosan used and the variations of durability testing from different labs.

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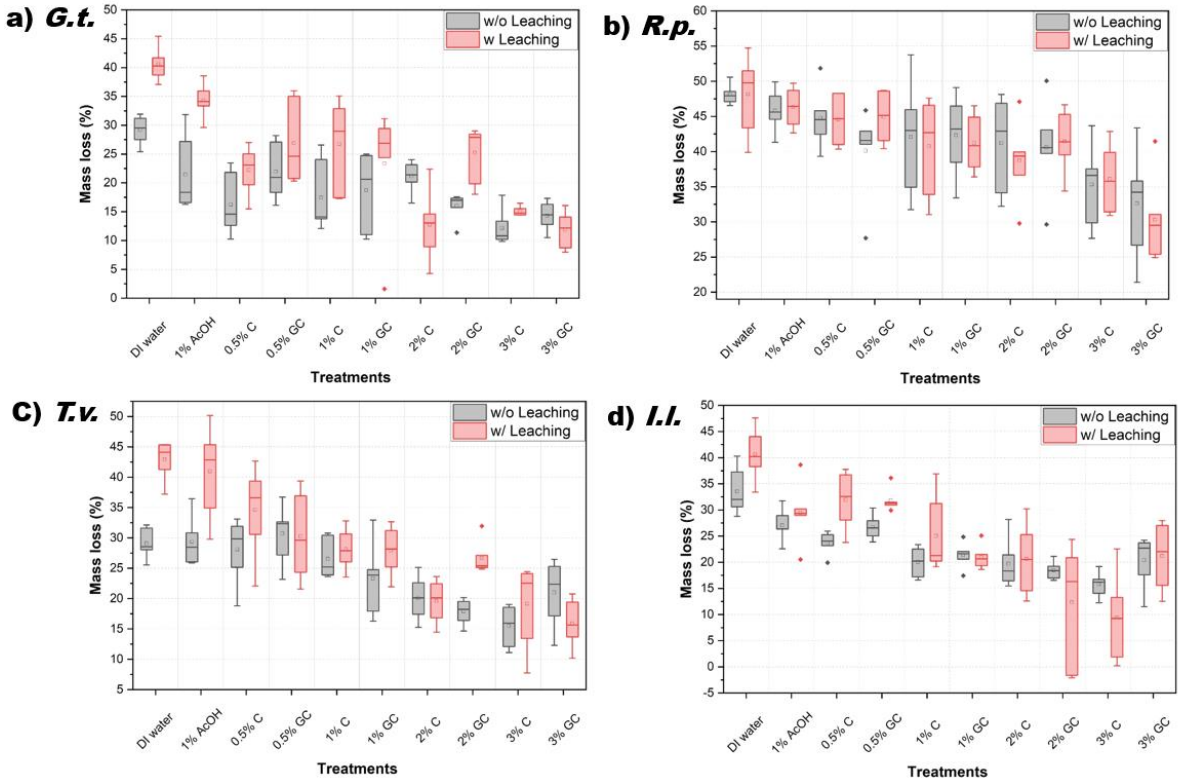

Figure 4. 8**:** Mass loss of water treated and preservative-treated wood with and without leaching after 4- weeks exposure to brown rot fungi; a) *G.t.* b) *R.p.* and white-rot fungi c) *T.v.* and d) *I.l.*

Chapter 5: Conclusion and Future Research Recommendation

5.1 Conclusion

This study demonstrated the feasibility of using genipin cross-linked chitosan for wood protection with a focus on reducing the leachability of chitosan. The cross-linking of genipin with chitosan was supported by FTIR and TGA characterization methods. Results from the *In vitro* study revealed GC is as effective as chitosan in inhibiting the growth of two common brown-rot fungi, *Gloeophyllum trabeum* (*G.t.*) and *Rhodonia placenta (R.p.)* and two white-rot fungi, *Trametes versicolor (T.v.)* and *Irpex lacteus (I.l.)*. *G.t.* was the most sensitive to the effect of C and GC treatment solutions across all concentrations. C and GC effect on *R.p.* fungi were also determined as fungistatic rather than fungi toxic. Microscopic analysis revealed GC treatment caused morphological changes and nuclei deformation of all the tested fungi.

After impregnating treatment of wood samples, the retention and mass gain of GC-treated wood samples were increased as the function of treating concentrations, which were as high as 21 kg/m^2 and 3.6%, respectively. However, cross-linking chitosan with genipin seems did not help with reducing the leaching rate of chitosan as low concentrations of 0.5 and 1% C and GC treated samples recoding negative mass gain values after treatment indicating complete leaching of the treated solutions form wood. Although GC treated wood samples generally show a significantly lower mass loss than those of the control groups regardless of the leaching test, our mass loss results were significantly higher than previously reported, which is conflicted with previous reports findings. The findings from this current study confirms that, chitosan from different sources yield different results.

5.2 Future research recommendations

Future research should consider crosslinking genipin with chitosan with much lower molecular weight (< 50kDa) than those employed in this study. This may result in reducing the viscosity whiles increasing GC penetration and encrusting in wood after treatment and eventually result in reduction of the leachability of chitosan. Additionally, crosslinking genipin with chitosan from different sources and evaluating their leachability is highly

recommended. MW of GC should be performed by SEC-MALS methods to unequivocally determine the crosslinking between C and genipin. Other biobased options to reducing the leachability of chitosan should still be the focus of researchers.

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Appendix

A) Fungi growth rate on GC amended and unamended plates after 14 days incubation period

		Fungi Growth rate													
		Days													
Treatment	**Fungi**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Control	***G.t.***	0.0	0.3	2.6	7.1	15.0	21.8	30.6	39.6	50.8	63.3	76.1	82.7	92.1	100.0
Control	***G.t.***	0.0	0.7	3.8	9.4	18.8	27.4	35.6	42.6	53.5	64.1	77.5	83.5	92.5	100.0
Control	***G.t.***	0.0	0.8	3.4	8.3	16.9	25.0	34.8	42.1	52.2	66.0	79.8	83.8	95.0	100.0
Control	***G.t.***	0.0	0.8	3.0	7.3	15.6	22.6	31.5	39.4	48.7	61.7	74.0	81.4	92.8	100.0
Control	***G.t.***	0.0	0.7	2.2	6.5	14.1	22.1	32.5	40.7	53.2	66.5	80.2	88.0	93.5	100.0
0.1% C	***G.t.***	0.0	0.0	0.7	3.6	7.1	9.9	13.6	15.4	17.9	20.4	22.0	23.5	25.8	29.3
0.1% C	***G.t.***	0.0	0.0	0.9	3.5	8.7	8.4	12.4	17.2	20.0	23.1	26.1	27.7	29.6	31.8
0.1% C	***G.t.***	0.0	0.0	0.0	2.4	8.3	11.8	16.0	17.4	19.8	22.8	25.3	27.8	30.8	34.6
0.1% C	***G.t.***	0.0	0.0	0.9	3.4	6.5	9.7	14.7	16.6	20.0	23.5	26.4	28.3	31.5	35.9
0.1% C	***G.t.***	0.0	0.0	1.5	3.7	7.5	9.8	12.5	13.7	16.3	19.7	22.2	25.0	27.2	29.1
0.1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.5	0.7	1.0	1.1	1.9	2.7	3.2	3.4	3.4	3.5
0.1% GC	***G.t.***	0.0	0.0	0.3	1.1	1.7	2.5	2.6	4.6	6.1	7.1	8.0	9.0	9.7	11.1
0.1% GC	***G.t.***	0.0	0.0	0.7	4.7	7.8	11.5	15.1	18.1	21.9	25.2	28.8	31.1	34.0	39.2
0.1% GC	***G.t.***	0.0	0.0	0.7	3.3	7.0	9.8	16.0	14.8	18.8	21.5	24.2	26.7	29.8	34.0
0.1% GC	***G.t.***	0.0	0.0	0.5	2.3	6.2	8.7	12.8	13.4	16.6	19.0	21.5	23.1	26.5	29.6
0.5% C	***G.t.***	0.0	0.3	0.8	0.9	1.1	1.6	1.7	1.8	2.2	2.2	2.5	2.4	2.7	3.1
0.5% C	***G.t.***	0.0	0.5	1.6	2.5	2.6	3.1	3.6	4.2	4.6	5.3	6.0	6.0	6.6	7.1
0.5% C	***G.t.***	0.0	0.3	1.7	1.3	1.7	1.9	2.0	2.1	2.3	2.6	2.8	2.9	3.4	3.7
0.5% C	***G.t.***	0.0	0.4	0.8	1.3	1.6	2.0	2.3	2.9	3.4	3.6	3.8	4.4	4.7	5.1
0.5% C	***G.t.***	0.0	0.3	1.0	0.9	0.8	0.7	0.8	0.8	0.9	1.3	1.7	1.9	2.0	2.4
0.5% GC	***G.t.***	0.0	0.0	0.3	0.8	1.1	1.4	1.5	2.0	2.6	3.2	3.9	4.1	4.2	4.4

0.5% GC	***G.t.***	0.0	0.0	0.3	0.6	1.0	1.6	1.7	1.7	2.3	3.0	3.2	2.7	3.0	3.5
0.5% GC	***G.t.***	0.0	0.0	0.3	0.8	1.0	1.9	1.6	1.7	2.3	2.9	2.8	2.9	3.2	3.2
0.5% GC	***G.t.***	0.0	0.0	0.2	0.5	0.5	1.9	0.5	1.0	1.7	2.1	2.6	2.5	2.7	2.9
0.5% GC	***G.t.***	0.0	0.0	0.0	0.3	0.7	0.9	0.6	0.8	1.4	1.9	2.0	2.0	2.2	2.6
1% C	***G.t.***	0.0	0.0	0.3	0.4	0.4	0.5	1.0	1.0	1.3	2.0	2.0	1.8	1.9	2.1
1% C	***G.t.***	0.0	0.0	0.3	0.3	0.3	0.3	0.1	0.1	0.3	0.5	0.5	0.7	0.7	0.9
1% C	***G.t.***	0.0	0.0	0.0	0.7	0.8	0.7	0.6	0.6	0.6	1.0	1.3	1.5	1.7	2.1
1% C	***G.t.***	0.0	0.0	0.0	0.6	0.4	0.6	0.6	0.8	0.9	0.9	1.2	1.2	1.2	1.4
1% C	***G.t.***	0.0	0.0	0.6	0.4	0.3	0.4	0.3	0.8	0.8	0.8	1.2	1.3	1.3	1.5
1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.3	0.4	0.5	0.3	0.4
1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.2	0.3	0.3	0.3	0.4	0.8	0.6	0.7	0.7	0.7
1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.2	0.2	0.3	0.3	0.4	0.6	0.6	0.8	0.8	1.2
1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.2	0.4	0.6	0.6	0.7	1.0
1% GC	***G.t.***	0.0	0.0	0.0	0.0	0.3	0.4	0.5	0.6	1.0	1.0	1.2	1.8	1.9	1.9
Control	***R.p.***	0.0	0.6	4.8	10.3	18.2	39.9	46.7	61.6	76.6	84.6	91.1	97.8	100.0	100.0
Control	***R.p.***	0.0	0.3	3.2	10.2	20.8	32.4	39.5	55.4	70.4	75.7	88.4	89.8	92.2	100.0
Control	***R.p.***	0.0	0.2	4.5	12.2	19.7	36.4	44.2	59.8	71.1	76.4	90.0	97.1	98.7	100.0
Control	***R.p.***	0.0	0.2	3.3	10.3	17.6	34.7	42.8	59.1	74.6	80.1	82.9	87.1	95.4	100.0
Control	***R.p.***	0.0	0.4	3.4	10.3	22.2	31.3	39.3	56.5	76.0	80.7	88.1	88.6	100.3	100.0
0.1% C	***R.p.***	0.0	0.0	3.2	10.6	23.0	26.1	34.2	44.2	67.6	77.1	84.9	86.2	99.4	100.0
0.1% C	***R.p.***	0.0	0.0	1.1	7.2	12.8	18.8	27.3	38.5	63.1	76.0	81.1	85.0	99.4	100.0
0.1% C	***R.p.***	0.0	0.0	2.0	7.7	18.0	20.8	32.4	41.4	58.7	78.8	82.2	85.3	99.9	100.0
0.1% C	***R.p.***	0.0	0.3	3.9	9.4	16.3	22.1	33.8	44.2	60.8	82.2	79.3	84.7	100.6	100.0
0.1% C	***R.p.***	0.0	0.0	3.2	9.6	14.7	23.9	31.1	43.1	69.3	84.6	86.8	91.0	101.0	100.0
0.1% GC	***R.p.***	0.0	0.2	2.6	7.2	11.5	15.0	25.1	35.7	57.7	76.7	83.3	87.2	95.4	100.0
0.1% GC	***R.p.***	0.0	0.0	1.9	7.1	11.4	16.9	23.8	36.4	57.0	69.7	81.7	83.8	94.1	100.0
0.1% GC	***R.p.***	0.0	0.0	2.8	5.8	9.9	15.6	21.7	32.8	60.5	70.6	85.7	88.0	100.1	100.0
0.1% GC	***R.p.***	0.0	0.0	2.1	6.2	13.6	14.5	25.5	36.2	60.1	74.4	83.3	85.4	99.6	100.0
0.1% GC	***R.p.***	0.0	0.0	2.2	6.3	13.8	18.3	32.9	41.2	62.4	73.6	80.6	84.2	96.1	100.0

0.5% C	***R.p.***	0.0	0.0	0.7	3.3	8.5	14.3	21.6	29.7	41.5	57.9	67.7	76.0	85.6	100.0
0.5% C	***R.p.***	0.0	0.0	0.8	3.4	9.2	13.6	20.5	29.4	40.3	55.4	70.5	78.5	82.3	100.0
0.5% C	***R.p.***	0.0	0.0	0.7	3.6	10.0	14.7	19.9	28.6	38.0	57.5	72.8	74.6	82.4	100.0
0.5% C	***R.p.***	0.0	0.0	0.5	2.1	6.2	10.9	19.2	27.6	37.7	55.5	74.4	80.2	82.9	100.0
0.5% C	***R.p.***	0.0	0.0	0.6	2.4	8.1	13.0	19.7	30.3	39.3	57.0	68.7	78.7	83.1	100.0
0.5% GC	***R.p.***	0.0	0.0	0.5	3.6	4.9	8.6	14.9	24.7	39.1	52.0	72.3	77.8	83.5	100.0
0.5% GC	***R.p.***	0.0	0.0	1.0	4.3	8.0	11.6	19.1	30.1	38.8	55.7	72.3	76.7	84.2	100.0
0.5% GC	***R.p.***	0.0	0.0	0.8	0.8	0.9	5.8	12.4	15.3	31.5	47.9	60.2	78.6	81.4	100.0
0.5% GC	***R.p.***	0.0	0.2	1.8	5.1	12.3	15.7	20.9	35.6	44.9	66.4	77.2	80.7	87.5	100.0
0.5% GC	***R.p.***	0.0	0.0	0.8	2.6	8.6	13.2	21.8	34.3	47.4	62.9	80.0	85.5	94.9	100.0
1% C	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% C	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% C	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% C	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% C	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% GC	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% GC	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% GC	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% GC	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1% GC	***R.p.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	***T.v.***	0.0	1.7	7.7	20.8	42.6	66.8	93.8	93.8	96.1	100.0	100.0	100.0	100.0	100.0
Control	***T.v.***	0.0	1.4	6.9	19.3	41.7	64.2	87.9	94.3	97.2	100.0	100.0	100.0	100.0	100.0
Control	***T.v.***	0.0	1.7	8.6	22.0	43.9	68.9	91.0	93.3	96.3	100.0	100.0	100.0	100.0	100.0
Control	***T.v.***	0.0	1.4	7.9	20.4	42.8	67.4	89.6	95.8	98.4	100.0	100.0	100.0	100.0	100.0
Control	***T.v.***	0.0	1.6	7.6	19.3	40.6	68.0	86.9	93.6	96.3	100.0	100.0	100.0	100.0	100.0
0.1% C	***T.v.***	0.0	1.8	2.9	6.0	11.3	16.7	22.0	28.8	37.9	46.8	55.3	61.4	67.6	77.2
0.1% C	***T.v.***	0.0	1.0	2.3	5.2	9.5	15.4	23.5	30.9	41.1	52.8	63.4	71.9	74.0	80.7
0.1% C	***T.v.***	0.0	1.3	2.9	5.6	10.4	17.0	25.4	32.4	37.9	40.9	42.8	43.1	44.3	44.4
0.1% C	***T.v.***	0.0	1.1	2.3	4.6	8.7	14.6	21.7	31.3	43.1	57.2	70.1	78.9	85.7	91.8

0.1% C	***T.v.***	0.0	1.5	3.2	6.4	11.3	16.9	25.4	33.5	45.7	58.7	69.4	74.6	82.9	88.1
0.1% GC	***T.v.***	0.0	1.3	4.4	9.5	17.4	27.2	37.2	47.4	63.0	74.9	83.9	89.2	95.6	100.0
0.1% GC	***T.v.***	0.0	0.5	4.1	9.5	18.3	31.7	42.0	55.1	73.2	90.7	97.9	98.2	98.4	100.0
0.1% GC	***T.v.***	0.0	6.6	4.8	5.7	9.6	17.4	24.6	25.3	37.3	45.8	59.9	69.4	85.7	100.0
0.1% GC	***T.v.***	0.0	1.3	2.8	6.3	12.5	21.8	32.2	44.3	59.9	73.5	89.4	97.8	98.8	100.0
0.1% GC	***T.v.***	0.0	1.1	4.3	8.1	14.2	26.0	30.3	38.7	49.9	59.3	73.2	82.4	89.7	100.0
0.5% C	***T.v.***	0.0	1.3	1.4	1.8	2.9	8.2	11.3	13.0	19.6	22.6	27.5	29.8	33.1	38.3
0.5% C	***T.v.***	0.0	1.1	1.4	2.0	2.3	6.0	9.3	17.6	15.8	18.3	22.2	23.1	26.1	27.1
0.5% C	***T.v.***	0.0	0.9	1.5	1.7	3.4	10.1	13.0	14.1	24.4	25.9	30.8	31.9	35.6	36.4
0.5% C	***T.v.***	0.0	1.2	1.2	1.5	3.4	8.7	12.5	14.4	18.8	23.3	26.1	30.3	31.4	32.9
0.5% C	***T.v.***	0.0	1.0	1.1	1.6	2.9	8.7	10.9	10.4	19.9	21.4	27.5	28.3	28.9	34.6
0.5% GC	***T.v.***	0.0	1.0	1.9	1.9	3.0	7.9	10.3	13.3	19.5	22.0	25.2	26.8	27.7	30.0
0.5% GC	***T.v.***	0.0	0.4	2.5	3.0	4.0	12.8	12.4	18.1	24.8	31.1	31.3	33.4	39.4	41.1
0.5% GC	***T.v.***	0.0	1.3	1.4	1.7	2.4	9.6	9.8	13.2	18.7	22.3	22.5	27.1	28.7	33.1
0.5% GC	***T.v.***	0.0	1.3	1.5	1.8	2.6	8.8	10.6	15.9	21.9	24.9	26.0	32.4	33.7	34.9
0.5% GC	***T.v.***	0.0	1.1	2.1	2.0	2.9	9.8	11.5	17.2	24.0	27.0	30.1	36.4	38.0	40.0
1% C	***T.v.***	0.0	0.9	1.1	1.7	2.7	3.2	5.2	5.7	6.6	8.3	9.3	9.8	13.6	14.9
1% C	***T.v.***	0.0	1.3	1.5	1.8	3.5	3.7	6.5	6.6	7.7	9.3	10.1	10.5	12.1	13.7
1% C	***T.v.***	0.0	1.1	1.4	2.1	3.3	3.8	6.4	7.6	8.9	10.8	12.3	13.6	14.2	15.4
1% C	***T.v.***	0.0	0.7	1.4	1.7	3.1	3.2	5.7	6.6	7.4	8.4	9.9	10.6	13.4	14.2
1% C	***T.v.***	0.0	1.0	1.6	1.8	3.2	3.3	5.7	7.1	9.8	10.7	13.3	13.8	16.2	17.3
1% GC	***T.v.***	0.0	0.0	0.4	0.6	0.8	2.0	2.9	3.5	5.8	5.6	7.5	7.9	8.1	8.9
1% GC	***T.v.***	0.0	0.0	1.0	1.3	2.0	4.6	5.0	7.1	8.6	8.8	8.4	10.5	11.0	12.6
1% GC	***T.v.***	0.0	0.2	0.9	1.2	2.5	4.9	5.8	6.9	8.1	8.7	10.5	11.3	13.8	16.1
1% GC	***T.v.***	0.0	0.1	0.6	0.9	2.1	4.5	5.6	6.5	7.8	8.1	9.2	10.7	12.7	15.4
1% GC	***T.v.***	0.0	0.5	0.9	1.4	1.8	6.4	5.7	6.8	10.1	11.5	12.7	15.5	15.8	17.7
Control	***I.l.***	0.0	2.6	15.5	39.4	68.1	84.5	96.6	94.7	96.7	100.0	100.0	100.0	100.0	100.0
Control	***I.l.***	0.0	2.8	15.0	36.8	74.6	85.6	96.9	95.1	99.7	100.0	100.0	100.0	100.0	100.0
Control	***I.l.***	0.0	2.3	13.9	37.1	75.4	87.6	96.0	96.0	99.9	100.0	100.0	100.0	100.0	100.0
Control	***I.l.***	0.0	2.0	13.2	34.5	71.0	82.9	95.4	95.2	99.8	100.0	100.0	100.0	100.0	100.0

Control	***I.l.***	0.0	2.2	13.7	35.2	73.4	84.4	96.1	95.7	98.9	100.0	100.0	100.0	100.0	100.0
0.1% C	***I.l.***	0.0	1.3	3.6	8.2	17.8	30.8	47.6	61.9	86.4	88.8	96.0	95.7	97.0	100.0
0.1% C	***I.l.***	0.0	0.9	4.3	10.3	20.0	30.9	42.7	59.4	80.8	83.6	93.8	97.0	99.2	100.0
0.1% C	***I.l.***	0.0	0.9	3.6	9.9	21.0	36.6	51.0	66.2	88.6	89.0	93.7	96.5	99.6	100.0
0.1% C	***I.l.***	0.0	1.0	4.1	10.5	17.5	28.4	35.1	52.9	83.4	86.9	94.7	97.6	94.2	100.0
0.1% C	***I.l.***	0.0	0.8	3.6	9.1	17.6	30.0	44.5	60.4	81.5	82.6	91.1	97.9	98.5	100.0
0.1% GC	***I.l.***	0.0	0.5	3.2	6.8	14.0	22.8	34.2	47.3	66.1	83.7	91.0	93.3	99.2	100.0
0.1% GC	***I.l.***	0.0	0.0	0.0	1.4	2.3	3.7	7.2	14.5	26.3	34.5	52.5	66.5	79.0	100.0
0.1% GC	***I.l.***	0.0	0.4	3.9	7.9	13.3	20.7	29.9	42.9	63.6	73.3	78.6	91.2	98.0	100.0
0.1% GC	***I.l.***	0.0	0.8	5.5	10.4	23.7	35.7	48.2	61.8	81.4	90.4	91.4	92.8	98.0	100.0
0.1% GC	***I.l.***	0.0	0.4	4.2	2.6	19.2	30.0	38.8	53.8	70.8	83.4	95.5	87.4	98.1	100.0
0.5% C	***I.l.***	0.0	0.8	1.0	2.7	3.8	7.7	16.5	16.6	23.8	26.7	27.6	28.6	33.9	39.4
0.5% C	***I.l.***	0.0	0.6	0.9	4.3	4.5	7.4	14.2	15.6	20.9	27.6	28.4	29.5	34.2	39.6
0.5% C	***I.l.***	0.0	1.7	2.7	3.2	3.6	9.6	10.1	10.7	14.8	20.6	24.1	27.0	27.7	32.8
0.5% C	***I.l.***	0.0	1.2	1.6	5.2	6.4	8.6	15.2	18.7	23.7	28.0	28.7	29.7	31.0	34.6
0.5% C	***I.l.***	0.0	1.1	2.1	3.7	4.0	8.7	11.6	15.1	22.5	28.0	28.4	30.7	32.3	37.5
0.5% GC	***I.l.***	0.0	1.2	1.7	1.1	2.4	7.5	8.8	12.8	18.3	22.0	23.9	25.3	30.4	36.3
0.5% GC	***I.l.***	0.0	0.8	1.4	1.0	1.6	5.7	6.2	8.1	9.5	11.8	13.3	15.4	17.7	21.5
0.5% GC	***I.l.***	0.0	0.9	1.5	3.3	1.9	6.8	8.2	11.4	14.9	16.9	20.2	23.1	30.0	31.7
0.5% GC	***I.l.***	0.0	1.6	2.0	2.2	2.6	5.4	8.9	15.0	19.1	21.5	27.8	31.6	37.3	42.9
0.5% GC	***I.l.***	0.0	1.2	1.2	1.4	1.4	4.2	6.0	8.9	12.0	13.2	14.6	16.0	17.9	21.2
1% C	***I.l.***	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.8	1.0	0.8	0.9	0.9	0.9
1% C	***I.l.***	0.0	0.0	0.0	0.0	0.0	0.7	0.8	3.8	5.8	6.6	7.2	7.5	7.9	9.8
1% C	***I.l.***	0.0	0.0	0.0	0.0	0.0	2.4	2.3	4.6	6.2	6.3	6.9	7.4	7.8	9.7
1% C	***I.l.***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.7	0.9	1.0	1.5	1.5	1.6
1% C	***I.l.***	0.0	0.0	0.0	0.0	0.0	2.5	2.5	3.3	4.4	4.6	6.5	10.3	10.3	10.7
1% GC	***I.l.***	0.0	0.3	0.5	0.9	0.8	1.0	0.6	0.9	1.0	0.9	0.8	1.0	1.3	1.3
1% GC	***I.l.***	0.0	1.1	1.1	1.2	1.7	1.8	1.8	2.0	2.4	3.4	3.6	3.9	5.0	5.9
1% GC	***I.l.***	0.0	0.9	0.5	0.8	1.0	1.0	0.9	0.8	1.1	1.1	1.1	1.3	1.3	1.9
1% GC	***I.l.***	0.0	1.0	0.9	1.2	1.3	1.4	1.1	1.3	2.0	2.0	2.0	2.0	2.0	2.1
1% GC	***I.l.***	0.0	0.7	0.9	1.2	1.3	1.3	0.9	1.1	1.6	1.7	1.5	1.5	1.5	1.5

B) Retention and Mass gain data for GC treated and untreated wood samples

W/O Leaching					W/ Leaching				
	Retention (kg/m3)		Mass gain (%)			Retention (kg/m3)		Mass gain (%)	
Treatment	**SW**	**HW**	**SW**	**HW**	**Treatment**	**SW**	**HW**	**SW**	**HW**
DI water	0.00	0.00	-0.76	-1.00	**DI water**	0.00	0.00	-0.26	-0.29
DI water	0.00	0.00	-0.74	-0.98	**DI water**	0.00	0.00	-0.31	-0.29
DI water	0.00	0.00	-0.79	-1.02	**DI water**	0.00	0.00	-0.32	-0.32
DI water	0.00	0.00	-0.87	-1.01	**DI water**	0.00	0.00	-0.33	-0.33
DI water	0.00	0.00	-0.92	-1.03	**DI water**	0.00	0.00	-0.34	-0.36
DI water	0.00	0.00	-0.91	-1.04	**DI water**	0.00	0.00	-0.33	-0.38
DI water	0.00	0.00	-0.92	-1.01	**DI water**	0.00	0.00	-0.36	-0.33
DI water	0.00	0.00	-0.91	-0.87	**DI water**	0.00	0.00	-0.39	-0.41
DI water	0.00	0.00	-0.94	-1.02	**DI water**	0.00	0.00	-0.40	-0.37
DI water	0.00	0.00	-0.93	-0.97	**DI water**	0.00	0.00	-0.39	-0.34
DI water	0.00	0.00	-0.95	-1.03	**DI water**	0.00	0.00	-0.39	-0.34
DI water	0.00	0.00	-0.97	-0.99	**DI water**	0.00	0.00	-0.34	-0.34
DI water	0.00	0.00	-1.01	-0.93	**1% AcOH**	0.00	0.00	-0.61	-0.59
DI water	0.00	0.00	-0.97	-1.01	**1% AcOH**	0.00	0.00	-0.73	-0.69
DI water	0.00	0.00	-1.01	-0.99	**1% AcOH**	0.00	0.00	-0.69	-0.72
DI water	0.00	0.00	-0.95	-0.96	**1% AcOH**	0.00	0.00	-0.68	-0.77
DI water	0.00	0.00	-0.92	-1.04	**1% AcOH**	0.00	0.00	-0.68	-0.71
DI water	0.00	0.00	-0.96	-0.91	**1% AcOH**	0.00	0.00	-0.68	-0.75
DI water	0.00	0.00	-0.97	-0.88	**1% AcOH**	0.00	0.00	-0.72	-0.69
DI water	0.00	0.00	-1.01	-1.02	**1% AcOH**	0.00	0.00	-0.70	-0.74
DI water	0.00	0.00	-1.03	-0.86	**1% AcOH**	0.00	0.00	-0.68	-0.67
DI water	0.00	0.00	-0.93	-0.89	**1% AcOH**	0.00	0.00	-0.73	-0.70
DI water	0.00	0.00	-0.94	-0.85	**1% AcOH**	0.00	0.00	-0.77	-0.72
DI water	0.00	0.00	-0.84	-0.73	**1% AcOH**	0.00	0.00	-0.70	-0.75
1 % AcOH	0.00	0.00	-0.28	-0.26	**0.5% C**	3.44	3.83	-0.49	-0.91
1 % AcOH	0.00	0.00	-0.29	-0.34	**0.5% C**	3.39	3.87	-0.47	-0.93
1 % AcOH	0.00	0.00	-0.31	-0.38	**0.5% C**	3.39	3.93	-0.50	-0.88
1 % AcOH	0.00	0.00	-0.26	-0.37	**0.5% C**	3.46	3.92	-0.52	-0.94
1 % AcOH	0.00	0.00	-0.28	-0.42	**0.5% C**	3.37	3.90	-0.54	-0.92
1 % AcOH	0.00	0.00	-0.31	-0.39	**0.5% C**	3.38	3.87	-0.60	-0.98
1 % AcOH	0.00	0.00	-0.34	-0.44	**0.5% C**	3.40	3.90	-0.57	-0.76
1 % AcOH	0.00	0.00	-0.30	-0.43	**0.5% C**	3.48	3.89	-0.56	-0.84
1 % AcOH	0.00	0.00	-0.26	-0.42	**0.5% C**	3.39	3.71	-0.59	-0.84
1 % AcOH	0.00	0.00	-0.19	-0.44	**0.5% C**	3.44	3.83	-0.58	-0.80
1 % AcOH	0.00	0.00	-0.07	-0.43	**0.5% C**	3.33	3.95	-0.56	-0.80

1 % AcOH	0.00	0.00	-0.22	-0.45	**0.5% C**	3.40	3.96	-0.49	-0.87
1 % AcOH	0.00	0.00	-0.19	-0.42	**0.5% GC**	3.37	3.98	-0.53	-0.64
1 % AcOH	0.00	0.00	-0.27	-0.37	**0.5% GC**	3.43	3.97	-0.52	-0.86
1 % AcOH	0.00	0.00	-0.24	-0.44	**0.5% GC**	3.40	3.98	-0.55	-0.77
1 % AcOH	0.00	0.00	-0.39	-0.55	**0.5% GC**	3.56	3.89	-0.62	-0.75
1 % AcOH	0.00	0.00	-0.35	-0.47	**0.5% GC**	3.55	3.94	-0.63	-0.74
1 % AcOH	0.00	0.00	-0.33	-0.46	**0.5% GC**	3.40	4.19	-0.57	-0.79
1 % AcOH	0.00	0.00	-0.33	-0.41	**0.5% GC**	3.62	4.17	-0.65	-0.75
1 % AcOH	0.00	0.00	-0.42	-0.45	**0.5% GC**	3.46	4.18	-0.61	-0.77
1 % AcOH	0.00	0.00	-0.40	-0.38	**0.5% GC**	3.56	4.04	-0.67	-0.77
1 % AcOH	0.00	0.00	-0.29	-0.42	**0.5% GC**	3.59	4.12	-0.62	-0.81
1 % AcOH	0.00	0.00	-0.31	-0.46	**0.5% GC**	3.62	4.21	-0.58	-0.85
1 % AcOH	0.00	0.00	-0.18	-0.48	**0.5% GC**	3.60	4.28	-0.56	-0.82
0.5% C	3.61	4.63	0.51	0.37	**1% C**	7.08	8.77	-0.19	-0.08
0.5% C	3.69	4.67	0.49	0.34	**1% C**	7.05	8.41	-0.18	-0.11
0.5% C	3.59	4.72	0.43	0.30	**1% C**	6.96	8.38	-0.15	-0.22
0.5% C	3.54	4.67	0.34	0.30	**1% C**	7.38	8.14	-0.10	-0.21
0.5% C	3.63	4.73	0.38	0.22	**1% C**	7.31	8.29	-0.12	-0.24
0.5% C	3.47	4.66	0.30	0.24	**1% C**	7.49	8.31	-0.13	-0.30
0.5% C	3.48	4.67	0.35	0.17	**1% C**	7.25	8.39	-0.16	-0.20
0.5% C	3.39	4.66	0.40	0.23	**1% C**	7.24	8.30	-0.14	-0.21
0.5% C	3.35	4.77	0.36	0.26	**1% C**	7.40	8.50	-0.13	-0.24
0.5% C	3.46	4.76	0.40	0.27	**1% C**	7.11	8.17	-0.15	-0.29
0.5% C	3.42	4.75	0.38	0.33	**1% C**	7.30	8.54	-0.16	-0.26
0.5% C	3.45	4.68	0.28	0.25	**1% C**	7.06	8.34	-0.26	-0.33
0.5% C	3.39	4.65	0.30	0.30	**1% GC**	7.15	8.40	-0.11	-0.02
0.5% C	3.42	4.68	0.43	0.35	**1% GC**	6.74	8.62	-0.21	-0.13
0.5% C	3.29	4.74	0.40	0.36	**1% GC**	7.03	8.64	-0.16	-0.20
0.5% C	3.42	4.67	0.34	0.38	**1% GC**	7.25	8.59	-0.15	-0.19
0.5% C	3.42	4.70	0.34	0.34	**1% GC**	7.17	8.83	-0.05	-0.15
0.5% C	3.55	4.68	0.35	0.30	**1% GC**	6.87	8.92	-0.14	-0.10
0.5% C	3.37	4.58	0.39	0.28	**1% GC**	6.98	8.55	-0.01	-0.31
0.5% C	3.52	4.67	0.41	0.35	**1% GC**	7.43	8.63	-0.18	-0.34
0.5% C	3.39	4.50	0.35	0.37	**1% GC**	6.82	8.48	-0.10	-0.36
0.5% C	3.42	4.50	0.45	0.36	**1% GC**	7.13	8.88	-0.12	-0.33
0.5% C	3.35	4.61	0.41	0.45	**1% GC**	7.17	8.90	-0.17	-0.34
0.5% C	3.45	4.56	0.46	0.54	**1% GC**	7.05	8.86	-0.22	-0.33
0.5% GC	3.36	4.54	0.39	0.39	**2% C**	14.25	17.91	0.70	0.95
0.5% GC	3.52	4.49	0.43	0.34	**2% C**	14.56	17.74	0.70	0.72
0.5% GC	3.36	4.53	0.33	0.34	**2% C**	14.23	17.99	0.73	0.73
0.5% GC	3.52	4.51	0.38	0.30	**2% C**	14.15	18.11	0.80	0.63

0.5% GC	3.43	4.49	0.33	0.28	**2% C**	15.08	18.40	0.80	0.77
0.5% GC	3.42	4.57	0.35	0.31	**2% C**	15.05	18.05	0.79	0.67
0.5% GC	3.35	4.56	0.31	0.33	**2% C**	13.96	18.06	0.70	0.81
0.5% GC	3.47	4.49	0.29	0.18	**2% C**	14.61	18.37	0.71	0.72
0.5% GC	3.39	4.55	0.33	0.30	**2% C**	14.05	18.09	0.69	0.57
0.5% GC	3.52	4.47	0.34	0.33	**2% C**	14.05	18.34	0.76	0.62
0.5% GC	3.58	4.51	0.33	0.36	**2% C**	13.77	17.97	0.69	1.01
0.5% GC	3.44	4.77	0.35	0.33	**2% C**	14.35	18.28	0.58	0.50
0.5% GC	3.48	4.77	0.37	0.38	**2% GC**	14.39	18.13	0.78	0.31
0.5% GC	3.53	4.74	0.37	0.32	**2% GC**	13.96	17.98	0.66	-0.33
0.5% GC	3.53	4.63	0.41	0.35	**2% GC**	14.43	18.42	0.70	0.39
0.5% GC	3.43	4.78	0.40	0.41	**2% GC**	14.34	18.61	0.74	0.58
0.5% GC	3.58	4.75	0.35	0.42	**2% GC**	14.35	18.51	0.71	0.54
0.5% GC	3.37	4.60	0.37	0.37	**2% GC**	13.57	18.25	0.56	0.59
0.5% GC	3.58	4.74	0.32	0.45	**2% GC**	14.45	18.14	0.76	0.66
0.5% GC	3.45	4.76	0.34	0.45	**2% GC**	14.06	18.02	0.72	0.55
0.5% GC	3.58	4.62	0.30	0.41	**2% GC**	14.44	18.16	0.69	0.54
0.5% GC	3.68	4.75	0.41	0.39	**2% GC**	14.93	18.16	0.72	0.50
0.5% GC	3.68	4.80	0.41	0.38	**2% GC**	14.76	18.21	0.73	0.43
0.5% GC	3.66	4.88	0.37	0.53	**2% GC**	14.46	18.15	0.70	0.42
1% C	7.32	10.03	1.02	1.17	**3% C**	20.67	26.89	1.45	0.56
1% C	7.47	10.06	0.93	1.20	**3% C**	20.45	26.74	1.39	0.26
1% C	7.54	9.58	0.86	1.11	**3% C**	20.57	27.40	1.38	1.47
1% C	7.09	9.67	0.78	1.16	**3% C**	21.22	27.28	1.50	1.35
1% C	6.87	9.65	0.80	1.09	**3% C**	20.79	27.69	1.62	1.58
1% C	7.17	9.68	0.88	1.30	**3% C**	20.65	27.67	1.57	1.67
1% C	7.11	9.82	0.85	1.31	**3% C**	20.67	26.93	1.36	1.57
1% C	7.11	9.55	0.87	1.27	**3% C**	20.87	27.04	1.43	1.49
1% C	7.01	9.53	0.90	1.16	**3% C**	21.47	27.08	1.39	1.29
1% C	7.44	9.28	0.96	1.26	**3% C**	19.99	26.88	1.35	1.28
1% C	7.29	9.39	0.95	1.29	**3% C**	20.59	26.70	1.36	1.23
1% C	7.55	9.57	0.88	1.18	**3% C**	20.74	27.43	1.35	1.15
1% C	7.45	9.46	0.80	1.38	**3% GC**	21.56	26.95	1.65	1.39
1% C	7.27	9.29	0.96	1.37	**3% GC**	22.70	27.56	1.73	1.47
1% C	7.06	9.48	0.99	1.34	**3% GC**	22.69	27.88	1.77	1.02
1% C	7.18	9.44	0.95	1.39	**3% GC**	22.22	26.98	1.69	0.53
1% C	7.58	9.37	0.95	1.24	**3% GC**	22.57	25.02	1.64	0.60
1% C	7.37	9.11	0.93	1.36	**3% GC**	23.55	26.57	1.72	1.25
1% C	7.26	9.36	1.04	1.29	**3% GC**	21.77	25.99	1.55	0.62
1% C	7.24	9.21	1.01	1.22	**3% GC**	23.31	26.24	1.64	1.15
1% C	7.37	9.30	1.04	1.26	**3% GC**	22.47	26.34	1.65	0.64

1% C	7.02	9.16	1.03	1.15	**3% GC**	23.41	25.60	1.56	0.08
1% C	7.21	9.47	1.07	1.22	**3% GC**	22.29	25.69	1.36	0.71
1% C	7.02	9.28	0.96	1.19	**3% GC**	23.06	25.77	1.36	0.94
1% GC	7.38	9.60	1.15	1.41					
1% GC	6.98	9.32	1.20	1.45					
1% GC	7.00	9.56	1.14	1.38					
1% GC	7.00	9.50	1.07	1.41					
1% GC	7.13	9.43	1.06	1.42					
1% GC	7.21	9.54	1.06	1.43					
1% GC	7.27	9.55	1.08	1.43					
1% GC	7.39	9.83	1.04	1.41					
1% GC	7.09	9.74	1.07	1.36					
1% GC	7.07	9.50	1.07	1.36					
1% GC	7.12	9.86	1.13	1.38					
1% GC	6.93	10.02	1.08	1.42					
1% GC	7.06	9.89	1.13	1.31					
1% GC	7.05	9.83	1.19	1.42					
1% GC	7.23	10.03	1.18	1.33					
1% GC	7.38	9.91	1.26	1.42					
1% GC	7.33	10.09	1.19	1.38					
1% GC	7.08	9.72	1.28	1.20					
1% GC	7.15	10.06	1.25	1.29					
1% GC	7.62	9.64	1.03	1.11					
1% GC	6.97	9.62	1.11	1.15					
1% GC	7.10	9.99	1.06	1.23					
1% GC	7.30	9.86	1.00	1.17					
1% GC	7.37	9.91	1.04	1.19					
2% C	13.75	19.64	2.29	3.17					
2% C	13.66	19.33	2.33	3.04					
2% C	13.69	19.45	2.43	3.07					
2% C	13.78	19.46	2.37	2.99					
2% C	13.37	19.28	2.21	2.86					
2% C	13.79	18.87	6.51	2.91					
2% C	13.50	18.87	2.27	2.98					
2% C	13.57	18.80	2.28	2.75					
2% C	13.36	19.16	2.35	2.74					
2% C	13.58	19.11	2.48	2.65					
2% C	14.04	19.36	2.36	2.94					
2% C	14.09	18.96	2.38	2.84					
2% C	13.90	19.15	2.49	2.68					
2% C	13.77	19.19	2.46	2.63					

2% C	13.89	19.15	2.37	2.66
2% C	13.63	19.05	2.34	2.36
2% C	13.72	19.34	2.32	2.93
2% C	14.04	18.91	2.30	3.11
2% C	13.57	18.89	2.31	3.08
2% C	13.86	19.29	2.30	2.95
2% C	13.36	19.22	2.41	2.70
2% C	13.42	19.42	2.52	2.77
2% C	12.94	19.34	2.51	3.33
2% C	13.36	19.48	2.37	2.75
2% GC	13.47	19.34	2.20	3.03
2% GC	13.40	18.89	2.18	2.86
2% GC	13.84	19.13	2.24	2.73
2% GC	13.30	19.29	2.27	2.57
2% GC	13.64	19.62	2.34	2.74
2% GC	13.20	19.17	2.35	3.43
2% GC	13.81	18.83	2.32	2.40
2% GC	13.14	18.95	2.24	1.62
2% GC	13.72	19.31	2.32	2.36
2% GC	13.50	19.35	2.39	2.68
2% GC	13.26	19.08	2.28	2.66
2% GC	13.02	18.85	1.99	2.74
2% GC	13.63	18.84	2.26	2.85
2% GC	13.62	18.91	2.18	3.00
2% GC	13.92	18.49	2.17	2.97
2% GC	13.78	18.69	2.21	3.15
2% GC	13.78	18.54	2.21	2.81
2% GC	13.84	19.20	2.30	2.98
2% GC	13.63	18.60	2.36	3.00
2% GC	13.48	18.78	2.51	2.78
2% GC	13.40	18.93	2.36	2.58
2% GC	13.80	18.77	2.38	2.66
2% GC	13.90	18.97	2.41	2.53
2% GC	13.70	19.22	2.40	2.61
3% C	19.29	25.07	2.97	3.80
3% C	19.08	23.45	3.05	3.45
3% C	19.07	23.81	3.01	3.59
3% C	18.41	25.28	3.08	3.91
3% C	19.08	26.22	2.77	4.00
3% C	19.24	24.17	2.84	3.73
3% C	19.23	18.04	2.97	2.38

3% C	19.16	16.05	2.88	1.93
3% C	19.29	26.20	2.95	3.85
3% C	19.84	25.59	3.05	3.80
3% C	19.43	26.14	3.32	4.25
3% C	19.15	26.31	3.24	4.21
3% C	19.29	20.69	3.06	3.05
3% C	19.09	25.19	2.91	4.12
3% C	19.36	25.89	2.83	4.03
3% C	19.22	25.57	2.93	3.98
3% C	19.22	26.23	3.18	4.21
3% C	19.15	25.34	3.16	4.40
3% C	19.04	25.91	2.98	4.34
3% C	18.75	26.15	3.01	4.22
3% C	19.23	25.61	2.92	3.89
3% C	18.49	25.69	3.04	3.97
3% C	19.09	25.64	2.99	4.02
3% C	19.15	26.06	3.04	3.95
3% GC	19.40	24.80	3.63	3.88
3% GC	19.63	21.41	3.73	3.34
3% GC	19.04	20.47	3.91	3.20
3% GC	19.33	23.16	3.74	3.69
3% GC	20.43	25.88	3.47	4.16
3% GC	20.73	25.47	3.59	4.20
3% GC	20.24	24.59	3.55	4.22
3% GC	20.67	26.04	3.53	4.23
3% GC	21.07	22.47	3.65	3.27
3% GC	20.73	18.22	3.58	2.57
3% GC	20.58	18.50	3.46	2.51
3% GC	20.89	23.75	3.48	3.54
3% GC	20.42	24.22	3.60	3.95
3% GC	20.89	20.19	3.69	2.89
3% GC	20.76	16.51	3.68	2.19
3% GC	20.72	16.72	3.77	2.14
3% GC	20.24	20.74	3.86	2.91
3% GC	20.29	14.33	3.69	1.65
3% GC	20.15	18.95	3.58	2.69
3% GC	20.92	22.97	3.59	3.56
3% GC	20.56	18.51	3.69	2.70
3% GC	20.61	14.61	3.38	1.80
3% GC	19.95	19.60	3.29	2.87
3% GC	20.32	23.89	3.30	3.48

Where: SW= softwood and HW= hardwood

C) Mass loss data of GC treated and Untreated control wood samples after fungi decay test

Treatment	Leaching test	*G.t.*	*R.p.*	*T.v.*	*I.l.*
DI water	**w/ Leaching**	31.19	47.80	32.14	32.78
DI water	**w/ Leaching**	27.50	46.56	31.63	40.31
DI water	**w/ Leaching**	28.18	48.07	28.17	37.28
DI water	**w/ Leaching**	25.42	48.54	28.95	28.77
DI water	**w/ Leaching**	31.93	50.59	25.54	31.23
DI water	**w/ Leaching**	30.83	47.11	28.05	30.63
DI water	**w/o Leaching**	41.68	54.73	37.20	38.31
DI water	**w/o Leaching**	39.83	49.36	41.28	39.93
DI water	**w/o Leaching**	38.72	51.49	44.76	47.60
DI water	**w/o Leaching**	37.09	50.20	45.38	33.41
DI water	**w/o Leaching**	45.43	39.90	45.33	40.49
DI water	**w/o Leaching**	40.74	43.36	43.48	44.04
1% AcOH	**w/ Leaching**	17.08	41.33	25.90	28.94
1% AcOH	**w/ Leaching**	16.59	46.03	28.70	26.36
1% AcOH	**w/ Leaching**	19.64	45.21	26.05	26.36
1% AcOH	**w/ Leaching**	16.27	44.60	28.23	31.74
1% AcOH	**w/ Leaching**	31.84	47.89	36.46	26.43
1% AcOH	**w/ Leaching**	27.18	49.91	30.83	22.59
1% AcOH	**w/o Leaching**	34.75	43.91	29.79	29.27
1% AcOH	**w/o Leaching**	29.61	49.73	34.92	28.97
1% AcOH	**w/o Leaching**	33.33	42.65	43.66	29.39
1% AcOH	**w/o Leaching**	33.44	47.27	45.34	38.63
1% AcOH	**w/o Leaching**	38.58	48.70	50.17	20.53
1% AcOH	**w/o Leaching**	35.93	45.57	42.02	30.21
0.5% C	**w/ Leaching**	10.26	51.84	18.79	23.65
0.5% C	**w/ Leaching**	14.79	39.33	25.16	19.91
0.5% C	**w/ Leaching**	14.37	45.80	29.57	24.44
0.5% C	**w/ Leaching**	12.62	44.10	30.10	25.25
0.5% C	**w/ Leaching**	23.47	45.01	33.11	25.96
0.5% C	**w/ Leaching**	21.82	42.46	31.90	23.16
0.5% C	**w/o Leaching**	19.67	46.89	30.57	37.77
0.5% C	**w/o Leaching**	26.99	48.29	22.06	28.47
0.5% C	**w/o Leaching**	22.71	40.99	42.66	36.67
0.5% C	**w/o Leaching**	25.05	40.36	36.08	36.71

0.5% C	**w/o Leaching**	15.48	42.46	39.36	23.81
0.5% C	**w/o Leaching**	23.50	48.29	37.13	28.07
0.5% GC	**w/ Leaching**	19.63	27.70	32.64	25.03
0.5% GC	**w/ Leaching**	16.10	45.87	32.59	26.27
0.5% GC	**w/ Leaching**	18.35	41.04	27.19	26.86
0.5% GC	**w/ Leaching**	22.29	41.52	36.72	30.35
0.5% GC	**w/ Leaching**	27.09	41.65	23.17	27.93
0.5% GC	**w/ Leaching**	28.21	42.91	32.13	23.89
0.5% GC	**w/o Leaching**	21.40	43.02	33.79	31.49
0.5% GC	**w/o Leaching**	27.83	47.31	39.39	29.92
0.5% GC	**w/o Leaching**	20.75	48.62	24.33	30.91
0.5% GC	**w/o Leaching**	20.33	40.41	36.95	31.19
0.5% GC	**w/o Leaching**	35.97	48.68	21.56	36.12
0.5% GC	**w/o Leaching**	35.03	41.56	25.45	31.35
1% C	**w/ Leaching**	14.20	45.97	24.30	17.20
1% C	**w/ Leaching**	13.75	44.13	30.78	23.36
1% C	**w/ Leaching**	13.98	34.91	23.90	19.53
1% C	**w/ Leaching**	12.08	31.75	26.15	16.62
1% C	**w/ Leaching**	26.59	53.75	30.43	22.52
1% C	**w/ Leaching**	24.07	41.85	23.64	20.92
1% C	**w/o Leaching**	35.06	31.04	28.57	36.92
1% C	**w/o Leaching**	32.90	33.89	32.79	21.69
1% C	**w/o Leaching**	29.15	39.61	23.54	20.85
1% C	**w/o Leaching**	28.75	45.76	26.06	20.23
1% C	**w/o Leaching**	17.42	46.60	27.19	31.25
1% C	**w/o Leaching**	17.27	47.58	30.64	19.15
1% GC	**w/ Leaching**	10.25	42.15	24.20	17.43
1% GC	**w/ Leaching**	11.04	44.27	17.95	22.07
1% GC	**w/ Leaching**	24.98	38.48	32.95	21.97
1% GC	**w/ Leaching**	20.88	49.11	23.56	21.26
1% GC	**w/ Leaching**	24.74	33.41	24.77	20.60
1% GC	**w/ Leaching**	20.41	46.47	16.29	24.86
1% GC	**w/o Leaching**	1.59	41.67	32.67	21.29
1% GC	**w/o Leaching**	26.90	37.83	29.03	19.36
1% GC	**w/o Leaching**	26.88	36.40	21.93	25.08
1% GC	**w/o Leaching**	24.37	46.52	31.22	20.00
1% GC	**w/o Leaching**	29.43	40.01	27.34	18.63
1% GC	**w/o Leaching**	31.14	44.91	25.22	21.51
2% C	**w/ Leaching**	22.61	48.11	21.05	17.71
2% C	**w/ Leaching**	20.16	46.87	15.29	16.46
2% C	**w/ Leaching**	24.04	34.13	25.15	18.96

2% C	**w/ Leaching**	23.16	32.22	19.17	15.49
2% C	**w/ Leaching**	16.52	42.72	17.44	28.18
2% C	**w/ Leaching**	20.16	43.08	22.61	21.39
2% C	**w/o Leaching**	14.61	29.79	14.46	14.54
2% C	**w/o Leaching**	12.80	39.97	20.82	20.36
2% C	**w/o Leaching**	4.28	36.63	16.83	20.70
2% C	**w/o Leaching**	8.93	38.77	19.43	30.21
2% C	**w/o Leaching**	13.31	47.10	22.45	12.59
2% C	**w/o Leaching**	22.39	39.93	23.63	25.29
2% GC	**w/ Leaching**	16.87	43.09	19.19	16.54
2% GC	**w/ Leaching**	17.35	29.64	19.53	16.97
2% GC	**w/ Leaching**	17.57	39.73	14.66	19.23
2% GC	**w/ Leaching**	11.37	41.10	16.41	18.53
2% GC	**w/ Leaching**	15.73	50.06	17.27	18.25
2% GC	**w/ Leaching**	17.09	40.02	20.17	21.13
2% GC	**w/o Leaching**	18.02	42.10	25.66	14.29
2% GC	**w/o Leaching**	19.84	45.30	25.15	20.88
2% GC	**w/o Leaching**	27.82	46.66	31.95	-1.63
2% GC	**w/o Leaching**	29.02	40.69	24.86	-2.10
2% GC	**w/o Leaching**	28.45	34.38	25.11	18.30
2% GC	**w/o Leaching**	28.01	39.55	27.12	24.37
3% C	**w/ Leaching**	10.22	27.66	12.12	16.35
3% C	**w/ Leaching**	10.48	29.87	11.10	12.24
3% C	**w/ Leaching**	17.85	37.55	18.56	16.73
3% C	**w/ Leaching**	9.87	43.67	19.04	14.04
3% C	**w/ Leaching**	13.35	37.48	18.42	16.09
3% C	**w/ Leaching**	11.14	35.74	13.50	19.19
3% C	**w/o Leaching**	14.47	31.43	23.24	1.87
3% C	**w/o Leaching**	15.54	39.90	24.45	22.56
3% C	**w/o Leaching**	14.60	37.60	24.11	5.31
3% C	**w/o Leaching**	14.97	30.91	21.83	0.21
3% C	**w/o Leaching**	16.48	33.98	13.45	13.21
3% C	**w/o Leaching**	14.44	42.86	7.74	13.28
3% GC	**w/ Leaching**	14.06	21.42	12.28	11.51
3% GC	**w/ Leaching**	12.80	26.70	17.15	17.60
3% GC	**w/ Leaching**	16.27	32.86	26.47	22.55
3% GC	**w/ Leaching**	10.51	43.38	25.31	24.23
3% GC	**w/ Leaching**	17.32	35.59	22.57	23.69
3% GC	**w/ Leaching**	14.88	35.81	22.19	22.85
3% GC	**w/o Leaching**	7.99	41.46	15.46	27.97
3% GC	**w/o Leaching**	8.71	31.08	10.19	24.37

3% GC	**w/o Leaching**	12.69	25.39	19.41	19.68
3% GC	**w/o Leaching**	14.08	30.47	20.75	27.04
3% GC	**w/o Leaching**	11.67	28.57	15.82	12.52
3% GC	**w/o Leaching**	16.07	24.92	13.70	15.58

C) SAS codes used for data analysis

i) Normality test

```
data Chitosan;
input treatment mass_gain;
      datalines;

;
proc print data= Chitosan;

proc univariate data= Chitosan normal;
        qqplot    mass_gain /Normal(mu=est sigma=est color=red l=1);
        by treatment;
        run;
```

ii) Homogeneity of variance test

```
data Chitosan;
input treatment mass_gain;
datalines;
;
proc anova data=chitosan;
class treatment;
model mass_gain = treatment;
means treatment;
means treatment / hovtest welch;
run;
```

iii) Game-Howell test

```
data Chitosan;

input treatment mass_gain;

datalines;

;

proc mixed data=Chitosan;
class treatment;
model mass_gain = treatment / ddfm=satterth;
repeated / group = treatment;
lsmeans treatment / adjust=tukey pdiff;
run;
```

iv) PROC GLM function

```
data Chitosan;
input treatment mass_gain;
datalines;
;
proc glm data=Chitosan;
class treatment;
model mass_gain = treatment;
means treatment;
lsmeans treatment / stderr pdiff lines;
run;
```