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Partial Purification and Characterization of Texas Live Oak (Quercus fusiformis) Lectin

Carmen Guadalupe Cruz

PARTIAL PURIFICATION AND CHARACTERIZATION OF TEXAS LIVE OAK (*Quercus fusiformis*) LECTIN

A Thesis

by

CARMEN GUADALUPE CRUZ

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

DECEMBER 2013

Major Subject: Biology

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ABSTRACT

Partial Purification and Characterization of Texas Live Oak (*Quercus fusiformis*) Lectin

(December 2013)

Carmen Guadalupe Cruz, B.S., Texas A&M International University;

Chair of Committee: Dr. Ruby A. Ynalvez

Lectins are carbohydrate-binding proteins with agglutination properties. There is a continuous interest in lectins due to their biological properties that can be exploited for medicinal and therapeutic purposes. The objective of this study was to isolate and characterize lectin in Texas Live Oak (*Quercus fusiformis*). More specifically, the study aimed to determine the lectin's blood group specificity and pH stability, determine effects of seasonal variation, soil moisture and soil pH on lectin activity. The study also aimed to determine the presence of antifungal activity in *Q. fusiformis* extracts. Lectin activity was detected and compared via agglutination and protein assays. Protein partial purification was accomplished using diethylaminoethyl ion-exchange chromatography matrix. High Performance Liquid Chromatography (HPLC) was used to assess purity of the lectin. Results showed that *Q. fusiformis* extracts' lectin activities are stable at a pH range of 5.2-9.2 but with a significant decrease in activity above pH 9.2. The lectin activity was significantly higher when assayed against sheep red blood cells as compared to other blood groups tested. *Quercus fusiformis* extract is devoid of antifungal activity against *Aspergillus niger* and *Rhizopus stolonifer.* The effects of seasonal variation, soil moisture and soil pH do not significantly correlate with lectin activity. Results from HPLC showed presence of three peaks indicating a partial purification of the *Q. fusiformis* lectin.

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INTRODUCTION

Lectin is a protein of non-immunological origin and contains at least one non-catalytic carbohydrate-binding site. Lectins' ability to recognize and specifically bind reversibly to carbohydrates distinguishes them among from proteins (Charungchitrak *et al*., 2011; Peumans and Van Damme, 1995). Their capability of specifically recognizing and binding to sugars of erythrocytes *in vitro* merits the term agglutinin (Peumans and Van Damme, 1995; Hajtó *et al*., 2005), the process is termed agglutination. Lectin's specificities such as binding to glycoprotein receptors on cellular membranes allow the study of the physiological means in cellular communication such as cell-to-cell recognition (Gonzalez de Mejía and Prisecaru, 2005). Thus, lectins play a pivotal role in cellular communication and protein-carbohydrate interactions (Sharon and Lis, 2004; Nagre *et al*., 2010). Lectin's sugar-binding property readily distinguishes them as biotechnological tools to define carbohydrate structure and physiological dynamics (Smets and Van Beek, 1984). This characteristic is exploited for biomedical research for wide applications (Van Driessche, 1998). Sources of lectins vary from organism to organism in terms of their function, structures, biological activity, concentration, as well as in organ and cellular location (Chrispeels and Raikhel, 1991).

Isolation and purification of lectin may be done through a variety of protein purification methods. Methods for purifying lectins vary due to lectin sources (i.e. plant or animal). Lectins are vast in structure, specificity, physiochemical properties and biological activity (Kuku *et al*., 2009). Also, combinations of protein purification methods vary. For example, in studying seeds of the pepper plant, *Capsicum annum* lectin was isolated and purified by a four-step procedure (Kuku *et al*., 2009). The four-step protein purification procedure for *Capsicum annum* included,

This Thesis is modeled on the International Journal of Biological Chemistry.

lyophilized (freeze-dried) samples and purification using two column chromatography techniques. The first column technique was with the ion exchange method. For this technique the *C. annum* samples were purified with two separate columns, DEAE-Cellulose and further purified with a second column, QAE-Sephadex. Lastly, purified *C. annum* samples were subjected to the second column chromatography method, affinity with a Sephadex G-100 column (Kuku *et al*., 2009). Another method previously used was the extraction of lectin from fresh leaves of *Kalanochoe crenata*. Adenike and Eretan (2004) isolated and purified lectin in a two-step procedure, one ion-exchange column chromatography running DEAE-celluose and one gel-filtration column chromatography, using Sephadex G-100. A third example in lectin isolation and purification includes *Artocarpus camansi* Blanco seeds, which was a three-step procedure. Blanco seed lectin was isolated with the ammonium sulfate precipitation (salting out) technique. Isolated lectin was then dialyzed and applied to gel-filtration column chromatography using a Sephadex G-200 column (Occeña *et al*., 2007).

The objective of this study was to isolate, partially purify and characterize leaf lectin from Texas Live Oak (*Quercus fusiformis*). The Texas Live Oak is native to Oklahoma and in north central, central and southern Texas (Simpson, 1999; Tull and Miller, 1999). This study focused on conventional methods of protein purification, which included centrifugation and column chromatographic techniques. High Performance Liquid Chromatography (HPLC) assessed the purity of the lectin. The lectin was characterized in terms of its animal blood group specificity, pH stability, seasonal variation's effect on lectin activity, and soil moisture and soil pH effect on lectin activity. The antifungal property of the Q. fusiformis extract was also determined. The research study was significant since it contributed to the inventory of lectins found in plants. It also established an efficient and effective protocol to isolate and purify lectin

from *Q. fusiformis*.

REVIEW OF LITERATURE

Lectins

Lectins are a large and diverse heterogeneous group of proteins (Kuku *et al*., 2009). In order for a protein to be classified as a lectin it must meet certain criteria: 1) protein must contain one non-catalytic domain that specifically binds reversibly to a mono-or-oligosaccharide (Peumans and Van Damme, 1995), 2) protein does not alter the carbohydrate covalent structure (Dixon, 1981), 3) protein must be divalent or multivalent (Barondes, 1981), 4) protein is of non-immunological origin (Rüdiger and Gabius, 2001) and 5) protein displays an array of biological properties upon agglutination (Adenike and Eretan, 2004). Lectins' high level of affinity for oligosaccharides and glycoconjugates seems to be based on their multivalency, multiple binding sites that allows for higher-level specificity (Loris, 2002).

Lectins contain many related protein families that share the common feature of distinguishing carbohydrates (Loris, 2002). Lectin families are subdivided into primarily three categories: animal, plant and microbial (Sharon and Lis, 2004). For example, Drickamer (1988) proposed categorizing animal lectin into one of two primary structural families, C-type or Galectins. Today, animal lectin in research recognizes twelve structural families (Kilpatrick, 2002) of which five structural families are considered classic families (Loris, 2002): C-type (Drickamer, 1988), I-type (Angata and Brinkman-Van der Linden, 2002), Galectins (Kasai and Hirabayshi, 1996), Pentraxins (Tillett and Francis, Jr., 1930) and P-type (Dahms and Hancock, 2002) families, which may vary due to structural alignments (Loris, 2002; Gabius, 1997).

Plant lectins are distinguished into seven families; four of these families are major constituents in plant lectin while the remaining three families are considered small protein families. These plant lectins are distinguished into seven families based on occurrence, molecular

structure, carbohydrate-binding specificity, molecular evolution and assessment of the physiological role of each (Van Damme *et al*., 1998). Van Damme *et al*. (1998), distinguishes and organizes the seven plant lectin families into: legume lectins, monocot mannose-binding lectins, chitin-binding lectins, Type 2 RIP and related lectins, jacalin-related lectins, amaranthin lectin and cucurbitaceae phloem lectin. Fungal and bacterial lectins are currently under investigation. There are few reviews of fungal lectin, which are found primarily in Basidiomycetes (KaWagishi, 1995). The discovery of lectin activity in higher fungi has prompted research in other fungal sources like *Lactarius deterrimus* (Giollant *et al*., 1993) as well as bacterial lectin with studies mainly on *Escherchia coli* or *Pseudomonas aeruginosa* (Eshdat *et al*., 1978; Gilboa-Garber, 1982; Cioci *et al*., 2003).

Since lectins are proteins ubiquitously distributed in nature (Cavada *et al*., 1998), plant lectin is extensively studied due to its rich diversity and accessibility. In addition, plant lectin is a convenient resource to study due to its ease of isolation, its usefulness as a reagent for glycoconjugates in solution and ability to bind to cellular surfaces. Moreover, plant lectins vary in structure and specific activities (Laija *et al*., 2010). Some examples are root lectin from *Microgramma vaccinifolia* that expresses toxic effects and antifungal activity on *Fusarium* species (Albuquerque *et al*., 2010) and wild cobra lily tubers from *Arisaema flavum* that expresses potent and antiproliferative activities (Singh *et al*., 2004).

Plant lectins

First discovered in the late 19th Century by medical student Hermann Stillmark (1888) in search of the toxic principle in castor beans, lectins were recognized as a protein in plant extract that contained the ability to recognize and bind to sugars on the cellular surface of erythrocytes and were thus termed hemagglutinin or phytoagglutinin (Sharon and Lis, 2004, Peumans and

Van Damme, 1995). Stillmark's research in castor beans lead him to describe this toxic protein to have ricin-like properties, and since then, this toxic component has progressively gained interest. However, these particular proteins are capable of differentiating between erythrocytes through an array of blood types, thus requiring the search for a new term to identify such proteins, lectin (Boyd and Shapleigh, 1954). With time, lectin gained more attention, especially in the early 1960s with a major discovery using red kidney bean (*Phaselous vulgaris)* lectin, a phytohemagglutinin that possesses mitogenic activity (Nowell, 1960). Mitogenic activity is the induction of mitosis of the cell via stimulation of lymphocytes. The introduction of lectin attributing immunological activity has led to the proliferation of lectin research.

More recently, European mistletoe (*Viscum album* L.) lectin has gained interest due the discovery of a wide range of activities such as immune modulated activities and apoptosis of tumor cells *in vitro* (Elsässer-Beile *et al*., 2005). Mistletoe, *Phthirusa pyrilfolia* is distributed throughout the tropical areas of Southwest Asia, Africa and South America. *Pthirusa pyrifolia* lectin was studied and reported to express antimicrobial activity (Costa *et al*., 2010).

Leaf lectins

The isolation, purification and characterization studies in seed lectin and leguminous type plants occurred until the mid to late 1970s. Observations of different plant species, plant organs and intracellular location slowly transitioned to discovering plant lectins in vegetative tissue, leaves, stems, bark, bulbs, tubers and flower tissues (Nair and Das, 2000; Yeasmin *et al*., 2007; Rüdiger and Gabius, 2001). The distribution of lectin is widespread throughout a number of diverse plant species. It has been found that plant lectin appears abundantly in leaves, seeds and roots (Chrispeels and Raikhel, 1991; Rüdiger and Gabius, 2001). There is extensive research on the isolation, characterization and purification of lectins from leaves, as well as surveying lectin

activity in leguminous and non-leguminous organisms. Leaf lectin studies are cost-effective due to their accessibility. Four examples of leaf lectin isolated, characterized and purified will be as follows: two examples of non-leguminous lectin and two examples of leguminous lectin. The first non-leguminous organism to be investigated was a plant widely found in Africa (where study was performed), *Kalanochoe crenata. Kalanochoe crenata* leaf lectin was purified and partially characterized (Adenike and Eretan, 2004). The second example of a non-leguminous source is Mulberry leaves. Leaves were shown to contain two new N-glycolylneuraminic acid-(NeuGc) binding lectins that portrayed anti-bacterial activity (Ratanapo *et al*., 2001). The first leguminous leaf example is *Griffonia simplicifolia* lectin. In this study, seed and leaf lectin were compared on properties such as binding affinities and hemaggluntination properties (Lamb *et al*., 1983). The second leguminous leaf example is *Erythirna indica* leaves, self-proclaimed as the first characterized *E. indica* vegetative tissue and found to have high specificity toward D-galactose and its derivatives along with lactose (Konozy *et al*., 2002).

Physiological roles of lectins

The physiological function of plant lectins remains unclear (Peumans and Van Damme, 1995). There are two proposed roles extensively studied in plant lectins, the first is involved with plant defense mechanisms and the second with symbiotic nitrogen-fixing bacteria association (Sharon and Lis, 2004). Plants are immobile, in constant exposure to pathogens and predators (Peumans and Van Damme, 1995) and do not have an immune system, thus the first proposal hypothesizes lectins to provide the first line of defense (Naeem *et al*., 2007). Defense mechanism against invertebrates, higher animals and phytopathogenic mechanisms relies on physiological measures of the plant (Lis and Sharon, 1998).

Plant lectin defense mechanisms are believed to be derived from lectins' ability to

agglutinate and immobilize microorganisms (Peumans and Van Damme, 1998). The plant lectin defense role revolves around two evidentiary items, first, the presence of lectins at potential sites of invasion by infectious agents and second, the binding of lectins to various fungi and their ability to inhibit fungal growth and germination (Charungchitrak *et al*., 2011). Lectin defense mechanisms note a number of ways by which lectins can interact with molecules within and outside the cell for defense. The first example is water uptake; dry soybeans imbibe water and release lectin into the absorbed water (Fountain *et al*., 1977). This results in the presence of lectins in the vicinity of the germinating seed where interactions among potential pathogens may occur (Chrispeels and Raikhel, 1991). A second example is the consumption by predators, which disrupts plant cellular structure and contents. Lectin released by disruption targets glycoproteins in the gut and may inhibit absorption of nutrients, which retards development or lead to death (Chrispeels and Raikhel, 1991). There are several suggested mechanisms of lectin insecticidal actions; interactions with glycoconjugates on membrane surfaces of epithelial cells along the digestive tract may affect signaling pathways, transport processes and immune responses (Napoleão *et al*., 2011; Fitches *et al*., 2008). One particular example is glucose-mannose lectin from *Cavavalia ensiformis* (Con A) that reduces movement of nutrients and digestive enzymes across the peritrophic membrane of the blowfly *Lucila cuprins (*Eisemann *et al*., 1994).

A third example of lectin interaction in plant defense is fungal hyphae growth into plant tissue and the disruption of cellular compartmentalization, which results in the release of lectins that may inhibit further hyphae growth (Chrispeels and Raikhel, 1991). One particular example of inhibiting fungal growth is seen in *Archidendron jiringa* Nielsen lectin, the Jenkol bean (Charungchitrak *et al*., 2011). Apart from plant defense mechanisms, plant lectin may also act against bacteria as displayed in *Archidendron jiringa* seed lectin (Charungchitrak *et al*., 2011)

and *Eugenia uniflora* seed lectin (Oliveira *et al*., 2008).

The second proposed physiological role of plant lectin is lectin's association between leguminous plants and their symbiotic nitrogen-fixing bacteria. Bohlool and Schmidt (1974) suggest that extracellular root lectin recognizes bacteria for the purpose of establishing symbioses. Lectin distributed in root tissue, especially in root hairs is a site for rhizobial entry, which host the nitrogen-fixing bacteria. The bacterial and rhizobial relationship initiates nodule development in legumes (Chrispeels and Raikhel, 1991; Lerouge *et al*., 1990; Sharon and Lis, 2004). This is a process of the utmost importance in both the nitrogen cycle of terrestrial life and in agriculture (Lis and Sharon, 1998). Overall, Peumans and Van Damme (1995) recognized lectins as defense proteins.

Lectins' ability to recognize and specifically bind to sugars on cellular surfaces is a recognized physiological effect (Gonzalez de Mejía and Prisecaru, 2005). This ability allows lectins to act as recognition molecules that may interact and or mediate cellular and intra-cellular exchange that may play a factor in physiological processes in living organisms (Sharon and Lis, 2004). Presently, lectins are viewed as invaluable biotechnological tools for biomedical and therapeutic uses (Naeem *et al*., 2007).

Factors that affect lectin activity

This study aims to observe lectin in a plant source and specifically focus on leaves; there are many factors that may affect lectin activity. The most common factors that influence lectin activity are pH, temperature and ionic conditions (Naeem *et al*., 2007). Lectins are suggested to resemble other defense related proteins due to their features such as pH stability over a wide pH range and resistance to animal and plant proteases (Naeem *et al*., 2007). Some lectins are heat labile (Gonzalez de Mejía and Prisecaru, 2005; Hung *et al*., 2009). Environmental factors such as

temperature, seasonal variation, photoperiod and nutrient availability have been reported to affect lectin content (Muramoto *et al*., 1991; Nsimba-Lubaki and Peumans, 1986). Nutrients vary among lectin sources, but among plants, food supply is in the form of water, sun and soil nutrients. The availability and quality of food supply varies from region and location as well as plant origin. Nutrient uptake availability relies on soil moisture, pH and texture. Consequently, the environmental factors such soil moisture and pH along with their seasonal variability is studied in this experiment.

Isolation and purification of lectins

Protein purification is a sequential process intended to isolate a target protein from a mixture. The isolation of the protein is characterized and further purified, utilizing different properties of proteins. The design protocol should have in mind the goal of the experiment along with information or characteristics of lectin. The objective of isolation is to permit lectin to be purified from a mixture and then characterized. Isolation is the backbone of protein purification and varies in approach due to a broad array of organisms that contain lectin as well as their various distinctive features. Isolation and purification of lectin begins with the preparation of the homogenate then crude extraction.

Isolation of lectin is carried out through a combination of methods using a mortar and pestle and/or use of a laboratory blender in order to achieve disruption of cellular wall and membrane followed by addition of a buffer (acidic or basic) depending on the protein's stability. These techniques physically and chemically disrupt plant cells resulting in a solution called a homogenate. Homogenization is usually followed by centrifugation, in order to separate the cell debris as a pellet and the crude extract containing the protein molecules as a supernatant (Lam and Ng, 2011).

Isolation of plant leaf lectin is the easiest phase of lectin studies due to accessibility, affordability and capability of providing information on the target sample. Characterization of lectin provides a raw understanding the functions and interactions of lectin. Such characteristics and functions include but are not limited to, amino acid composition (Sharon, 1993; Martinez-Cruz *et al*., 2001); size, shape, net charge, isoelectric point (Pajic *et al*., 2002); solubility; carbohydrate-binding specificity with sugars such as, D-galactoside specific, galactose-specific, fucose-specific, mannose-specific (Kawsar *et al*., 2008; Sampaio *et al*., 1998; Pan *et al*., 2010; Van Damme *et al*., 2000), pH stability (Kuku *et al*., 2009); heat-stability (Ynalvez *et al*., 2011); and ligand/metal binding properties (Sampaio *et al*., 1998). These characterizations are advantageous to the experimenter because they may be exploited in formulating the protein purification protocol. Certain features may entail explicit or in-differential means to continue protein isolation. For example, the solubility of lectin involves a two-phase system supernatant (aqueous/liquid solution) and precipitate (protein), the two most common protein precipitation techniques are ammonium sulfate precipitation or polyethylene glycol (PEG). The ammonium sulfate precipitation technique isolates lectin through the addition of salt (Jakoby, 1971). Polyethylene glycol (PEG) is a non-ionic, water-soluble polymer and based on the degree of PEG concentrate, the desired concentration of lectin becomes fractionated (Ingham and Busby, 1980).

Another example of a lectin feature that may be exploited is its size and shape. Centrifugation is one of the most commonly used methods to precipitate lectin. In principle, centrifugation separates the mixture into cellular components but in protein purification it also provides bimolecular insight into lectin's size and shape. A protein constituent is relatively dense compared to cellular components such as organelles and hence precipitates to the bottom.

Protein purification can be a one-step or multi-step procedure. Increasing the number of steps may result in decreased overall protein recovery, which conversely increases protein purification success and alters overall functional activity (Janson, 2011). Variations in chromatographic techniques exist due to desired means of purifying protein according to differences in specific properties that are expressed in characterization and isolation; again some examples include solubility, size or charge.

A variety of chromatographic techniques are widely used, including ion-exchange chromatography (IEX), which relies on charge-charge interactions because it separates protein by exploiting their ionic net charge (Janson, 2011). Ion exchange columns are composed of beaded matrices that are charged; columns vary in ionic strength from weak to strong. Ion-exchange chromatography may be subdivided into anionic (positive) chromatography or cationic (negative) chromatography. Anionic chromatography is a positively charged column that attracts negatively charged protein molecules. Popular examples of anionic columns are Diethylaminoethane (DEAE) and Quaternary amine (Q-anion) (Janson, 2011). Conversely, cationic columns are negatively charged and bind positively charged protein molecules; for example, a Carboxymethyl (CM) column (Janson, 2011). The principle of ion-exchange chromatography is to optimize binding of target charged proteins by adsorbing the protein to the column to be bound, then eluted and washed out by a salt gradient concentration.

Another chromatographic technique is affinity (bio-recognition), which exploits carbohydrate/ligand/metal binding properties. For example, known ligands are monosaccharide or disaccharides, such as lactose, galactose, polysaccharide N- acetylglucosamine and a mannose polymer (Almanza *et al*., 2004; Konami *et al*., 1991; Chen and Xu, 2005; Argayosa *et al*., 2011). Analytical assessment in examining the purity of protein requires different and higher

functioning chromatographic techniques, such as High Performance Liquid Chromatography (HPLC). High Performance Liquid chromatography is a higher performing chromatography method because of its efficiency in analyzing its applied mixture into smaller particles that successfully improves the exposure of the sample for analysis and reduction of particles due to diffusion time (Bird, 1989). Overall, HPLC optimizes the separation of components and assesses protein purity. It allows for detection of low levels of proteins in mixtures. HPLC is a superior system that includes five main components, a pump, column, injector, detector and computer (Bird, 1989) that measures the amount of protein in a mixture and quantitatively analyzes the sample's components. The HPLC enriches protein by removing impurities and producing pure protein that may be used for future application studies (Johansen *et al*., 2009; Green *et al*., 1987).

Lectins' applications

Lectins' ability to bind specifically, not alter and have a higher affinity for oligosaccharides (not present in plant tissue) and glycoconjugates (present on the surface of bacteria and fungi) provides support that lectins may be exploited for diverse applications. As a result, lectin is investigated and observed for *in vitro* and *in vivo* studies. *In vitro* studies include biological activities, immunological purposes such as diagnostic and therapeutic uses, biochemical enhancements or binding specificity (Kaur *et al*., 2005). For example, lectin's binding specificity provides its ability to act as recognition molecules, which allows for physiological communication to take place in cellular processes (*in vivo*). Lectin may identify changes that occur on cellular surfaces during cell differentiation or recognize malignant cells (Sharon and Lis, 2004). As observed *in vitro*, lectin agglutinates with complex carbohydrates (Ray and Chatterjee, 1995), glycoproteins (Rambaruth *et al*., 2012), erythrocytes (Boyd and Shapleigh, 1954), lymphocytes, (Prujansky *et al*., 1978) fibroblasts (Sell and Paulino da Costa,

2003), fungi (Barkai-Golan *et al*., 1978) and bacteria (Patchett *et al*., 1991). As a result, lectins are labeled as biotechnological tools and used to examine biological activities such as anti-insect (Kaur *et al*., 2005 and Hossain *et al*., 2006), termiticidal (Napoleão *et al*., 2011), anti-microbial (Costa *et al*., 2010), anti-fungal (Kuku *et al*., 2009) and anti-HIV agents in the form of microbicides and virucides (Balzarini *et al*., 2004; Fang *et al*., 2010). Also, lectin has been found to be a tool in immunological studies such as anti-tumor (Arab *et al*., 2010), anti-carcinogenic (Gonzalez de Mejía and Prisecaru, 2005), antinocieptive effects (Leite *et al*., 2012), lympho mitogenic (Nagre *et al*., 2010), anti-inflammatory (Assreuy *et al*., 1997) and pro-inflammatory (Alencar *et al*., 2005), smooth muscle relaxant effects (Lima *et al*., 2010) and anti-neoplastic (drug deliveries) (Poiroux *et al*., 2011; Liu *et al*., 2010; Li *et al*., 2011).

More specifically, leaf lectins were reported to express antifungal activity such as with *Manihot esculenta* leaves (Silva *et al*., 2010); *Schnius terebinthifolous* leaves, a Brazilian pepper tree (Gomes *et al*., 2010); and *Indigofera suffruticosa* leaves which report both anti-bacterial and anti-fungal activities due to lectin presence (Leite *et al*., 2006).

Texas live oak

The Texas Live Oak (*Quercus fusiformis*) is a thicket-forming shrub (or a large spreading tree) and is a drought-tolerant and cold-hardy plant compared to its look-a-like *Quercus virginiana.* The Texas Live Oak is native to Oklahoma and in north central, central and southern Texas (Simpson, 1999; Tull and Miller, 1999). Texas Live Oak, *Quercus fusiformis* belongs to the family Fagaceae and may also be known as Escarpment Live Oak, Plateau Live Oak, Scrub Live Oak and West Texas Live Oak (Simpson, 1999). The leaves of Texas Live Oak are evergreen, with a firm texture, ovate to elliptic, 1 to 6 inches (2.5-25 cm) long and usually do not contain lobes except in young plants. The fruit produced by Texas Live Oak are acorns that are

½ to 1 inch (1.2-2.5 cm) long, spindle-shaped and narrow at the base. Several native plant species of Texas and south Texas region have yet to be examined as potential lectin sources, with the exception of Texas Live Oak. Ynalvez *et al*., (2011) reported lectin to be present in the leaf, stem and fruit of *Q. fusiformis* with the highest lectin activity expressed in the leaves.

METHODOLOGY

Sample collection

Leaf samples were collected from Texas Live Oak trees found on the Texas A & M International University campus. There is an abundance of these trees on campus; therefore five trees were selected to sample and represent the Texas Live Oak tree population (N27º34'24.3" W99º26'00.3"; N27º34'23.4" W99º26'12.3"; N27º34'22.3", W99º 26'17.3"; N27º34'34.5" W99º25'49.0"; N27º34'23.3" W99º25'59.1). Leaf samples collected were mature sized leaves that ranged from 8 centimeter to 12 centimeter in size. Leaf samples were stored in assigned labeled Ziploc™ bags placed in a -40ºC freezer or immediately homogenized.

Crude extraction

Leaves were washed, ground and homogenized using a Waring® Laboratory variable-speed blender. The leaves were blended for one minute until leaf veins were apparent. Blending was continued until leaves appeared as a powder. Subsequently, cold 0.01 M Trizma buffer (0.15 M NaCl), pH 9.4 (1:8 w/v) was added and homogenized for one minute for each respective sample. Samples were stirred for one hour in a cold room (4˚C) to optimize homogenization. Then, the homogenate was filtered using cheesecloth and centrifuged at 8,000 rpm at 10°C for 30 minutes using an Avanti® JE Centrifuge JA-20 Rotor. The crude extract was stored (at -40˚C) for later use or immediately used for agglutination assays.

Preparation of red blood cells

Two-hundred microliter aliquot of blood samples including human (collected from Laredo Medical Center Laboratory), horse, rabbit and sheep (purchased from Biomérieux® company) were mixed with 10 mL 0.01 M, phosphate buffer saline (PBS), pH 7.2 (0.15 M NaCl) in a 15 mL tube and centrifuged using a Hamilton Bell® VanGuard V6500 Biohazard centrifuge

for five minutes at full speed. At the end of centrifugation, the supernatant was discarded. A second wash included 10 mL of 0.01 M, PBS or until the supernatant was clear to remove lysed red blood cells. The pellet was resuspended with PBS to obtain a 2% blood suspension.

Agglutination assays

The crude extract and purified fractions were assayed for the presence of lectin activities using Corningware™ 96-well microtiter U-plates, 0.01 M PBS and 2% blood suspension. The sample was diluted by a serial two-fold dilution in PBS (50 µL) and incubated with a 2% suspension of RBCs (50 µL) at room temperature. The microtiter plate was left undisturbed for an hour until the negative control showed a red button formation. Agglutination activity was detected based on the appearance of the well; a positive result appears as a red-carpet layer, while negative results, appear as a red button in the bottom of the well.

Fig. 1: Microtiter plate row showing results of an agglutination assay. The positive agglutination results appear as a red-carpet layer, while negative results appear as a red button

Protein content determination

The protein content of crude extract samples were determined with the Bradford method (Bradford, 1976) using a QuickStart™ Bradford Protein Assay Kit 2, which contains bovine γ-globulin (BGG) reagent and seven standard concentrations (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml). Protein concentration was measured using a UV Spectrophotometer at a wavelength of 595 nm and disposable cuvettes. First, standards were prepared with two cuvettes prepared for 20 µl each standard concentration. Two controls were prepared and contained only 0.01 M PBS (0.15 M NaCl) to calibrate the UV Spectrophotometer at 595 nm. Each sample was prepared

with 20 µl of crude extract and assayed in three trials. After 1 mL of Bradford reagent was added to each cuvette, the cuvettes were vortexed for 30 seconds. Lastly, cuvettes were incubated at room temperature for 15 minutes and curvettes were read at wavelength, 595 nm.

DETERMINATION OF LECTIN ACTIVITIES

Blood group specificity

Lectins exhibit specificity towards a specific animal blood group and may or may not bind to a specific blood type. The blood group specificity was tested in four different bloods; horse, human, rabbit and sheep. The animal blood was purchased as defibrillated blood from selected suppliers at Biomérieux®. Human red blood cells were collected from the Laredo Medical Center (LMC) Laboratory Department, as postpartum blood samples and screened for negative results in both HIV and blood-borne communicable diseases. *Quercus fusiformis* lectin was tested for blood group specificity based on enhanced or decreased agglutination activity.

Effect of pH on *Q. fusiformis* lectin activity

Crude extracts of *Q. fusiformis* were studied for the effect of pH by using buffers of different pH levels namely acetate buffer, phosphate buffer saline and Trizma-HCl buffer. The effect of pH determined the optimum pH of the lectin under study. The buffers pH ranges from 5.2-9.2. For pH 5.2 buffer, acetate buffer was used, while for pH 6.2 and 7.2 phosphate buffer was used. For pH levels of 8.2 and 9.2, Trizma-HCl buffer was used. The buffers were chosen based on their pKa values.

Effect of seasonal variation on *Q. fusiformis* lectin activity

Leaf samples were collected in three seasons (fall, winter and summer) to determine if seasonal variation had an effect on *Q. fusiformis* lectin activity. Crude extracts of *Q. fusiformis* were prepared with 0.01 M Phosphate buffer saline (0.15 M NaCl), pH 7.2. Agglutination assays were performed with available rabbit blood to detect lectin activity.

Effect of soil moisture on *Q. fusiformis* lectin activity

The effect of soil moisture was assessed using soil samples collected by the auger

method. Soil was sampled and subsampled by depth. Soil nutrient availability is influenced by soil moisture, soil pH and soil texture; however, this study only examined the effect of soil moisture and pH (see below). Soil examined was under the *Q. fusiformis* crown and collected from three *Q. fusiformis* locations.

A hand auger was used to collect a composite of five samples from each tree at three separate depths: 0-10 centimeters, 10-20 centimeters and 20-30 centimeters. Samples were stored in a cold room (4˚C) or used immediately. A composite of five samples was consolidated by three separate measurements. Soil moisture was determined by examining moisture loss of soil subsamples on aluminum weight trays. Each depth (0-10 centimeters, 10-20 centimeters and 20-30 centimeters) was investigated separately. Samples were weighed and recorded using an analytical balance. A moist, 2.00 gram subsample was used for each triplicate subsample. Soils were oven-baked for 24 hours at 105˚C to dry and weighed once more to record dry soil mass.

Effect of soil pH on *Q. fusiformis* lectin activity

Soil samples collected were used to determine soil pH. Soil pH was observed for consistency between sampling sites. The Laredo region has silty clay loam soils (USDA, 1985) and typical south Texas extreme temperatures that are suboptimal conditions for the Texas Live Oak, during hot summer months. Soil pH may also influence water quality and soil nutrients needed for growth of Texas Live Oak which in turn influences lectin production.

Soil samples were deposited in paper bags to air-dry for three to seven days, followed by sieving with a two-millimeter (mm) mesh screen (#10 mesh). Next, a collection of 5.00g air-dry soil samples was placed into a 50 mL labeled centrifuge tubes. A total of three replicates were performed for each soil depth collected: 0-10 centimeter, 10-20 centimeter and 20-30 centimeter. Ten mL of deionized water was added to the 50 mL centrifuge tube for a soil water ratio of 1:2.

Solutions were shaken for one hour at 120 revolutions per minute (rpm); subsequently, solutions were centrifuged at 15,000-x g for 5 minutes. The resulting supernatant was used to record pH using the VWR® sympHony pH meter.

Determination of the antifungal activity of *Q. fusiformis* extracts

Potential fungal inhibition activity was evaluated by preparing two antifungal assays, an anti-*Aspergillus niger* and an anti-*Rhizopus stolonifer* assays. Potato Dextrose Agar (PDA) plates were marked with a circular circumference on the bottom of the plate with a 60 millimeter (mm) perimeter. Fungal PDA plates were prepared for optimal fungal growth with incubation for 23 hours at 28ºC. Fungal cultures were grown until they reached the drawn boundaries. After the mycelial colony had grown, sterile blank paper discs (6mm in diameter, Grade AA) were saturated with 20 µL aliquot solutions of *Q. fusiformis* freeze-dried crude extracts that were diluted with millipore water. The concentrations were as follows 125 µg/µl, 250 µg/µl and 500µg/µl. The discs were placed at the outer rim of the mycelial colony (which was within the new circumference of 60 mm away from the original circumference of the petri plate). The plates were incubated at 28ºC for 23 hours. After incubation, crescents of inhibition were measured using a vernier caliper. A negative control was used (Dimethly sulfoxide, DMSO) which allowed mycelial growth on envelope discs containing the control. In addition, a positive control (nystatin) was used to demonstrate the formation of crescents of inhibition around the disc.

Purification of *Q. fusiformis* lectin

The lectin from *Q. fusiforms* crude extracts was purified by one step chromatography using an ion-exchange column chromatography using a Bio-Rad® BioLogic™ Low-Pressure Chromatography system with BioFrac Fraction Collector (#731-8304). For *Q. fusiforms,* 26 mL of crude extract was applied to DEAE-cellulose column, which was equilibrated and washed

with Buffer A 0.01 M Trizma-HCl, pH 9.2 (0.15 M NaCl) at a flow rate uniform for all buffers (1ml/min collecting 3mL for each fraction). After washing, a one-step up salt gradient of 0.5 M NaCl to 1.0 M NaCl (Buffer B and Buffer C, respectively) eluted and desorbed bound proteins. The elution profile of Buffer B, 0.5 M NaCl, was read at absorbance 280 nm and eluted protein until absorbance was consistent and read below < 0.01, then elution Buffer C was applied. A Bio-Rad® Fraction Collector was used to collect fractioned proteins. Fractioned proteins were pooled and dialyzed to remove the salts.

Dialysis

Dialysis was performed with Thermo Scientific SnakeSkin® Dialysis Tubing, 7K membranes and used as a traditional form of dialysis that allows desalting and reverse basic pH 9.4 to neutral pH 7.4 and buffer exchange for 10 to 100 ml samples. For best results, a recommended dialysate volume of at least 200-fold greater than sample volume was used (high buffer-to-low sample volume). The dialysate used was deionized water. SnakeSkin® tubing was first calculated for by formulating the amount of Snakeskin tubing needed based on the volume of fractions pooled. The Snakeskin® tubing was pre-washed in running deionized water for 30 minutes to remove cellulose. Fractions were sealed with Thermo Scientific® clips to float and suspended in each of their according beaker of dialysate, which stirred for two hours in the cold room (-40^{o}C). After stirring for two hours the dialysate was discarded and replaced with 200-fold deionized water (DiH_2O) and stirred another 2 hours. For the third time, the diH_2O was discarded and replaced with diH_2O and left to stir overnight. Upon completion, diH_2O was discarded and samples were measured for pH with litmus paper. The sample was at pH 7.4 and was subjected to an agglutination assay with rabbit blood.

Determination of *Q. fusiformis* purity via HPLC

Purity assessment of *Q. fusiformis* was analyzed through a Varian® ProStar HPLC using a RR-C-18 matrix column (250 x 4.60mm) equilibrated at a flow rate of 1.0 mL per minute, UV absorbance of 335 and two primary solvents methanol (solvent A) and water (solvent B). Elution profiles were monitored at 215 nm to 280 nm with analytical software Galaxie© Software. HPLC was equilibrated through a pre-run of linear gradient of 100% to 0% of methanol and a 0% to 100% of water, respectively. In order for HPLC to analyze samples, 2 µL of freeze-dried samples were needed and diluted in 100 µL diH_2O.

Statistical analysis

Data for blood group specificity and pH stability was carried out in five replicates. Statistical Analysis System® with a 5 x 4 factorial experiment in randomized complete blocks design with mean comparisons was used to analyze significant differences by a Bonferroni test using a type I error rate of 5%. Data analysis for seasonal variation was analyzed in a one-way analysis of variance (ANOVA). Seasonal variation, soil moisture and pH data analysis was carried out in triplicates and presented as a correlation analysis with level of significance for a two-tailed test with level 0.01 as being statistically significant.

RESULTS AND DISCUSSION

Lectin activity from *Q. fusiformis* leaves was investigated, using agglutination assays. In the presence of lectin, sugars on the surface of red blood cells form an interaction with the lectin resulting in agglutination. This is evident by the formation of a carpet layer on the bottom of a microtiter plate wells (Figure 2, wells 1-3 or 1-4 indicated by arrows). On the other hand, in the absence of lectin, sugars on red bloods cells interact among themselves and form a distinctive red button on the bottom of the microtiter plate well. The reciprocal of dilution is calculated as titer value, which reflects lectin activity, the higher the value the higher lectin activity.

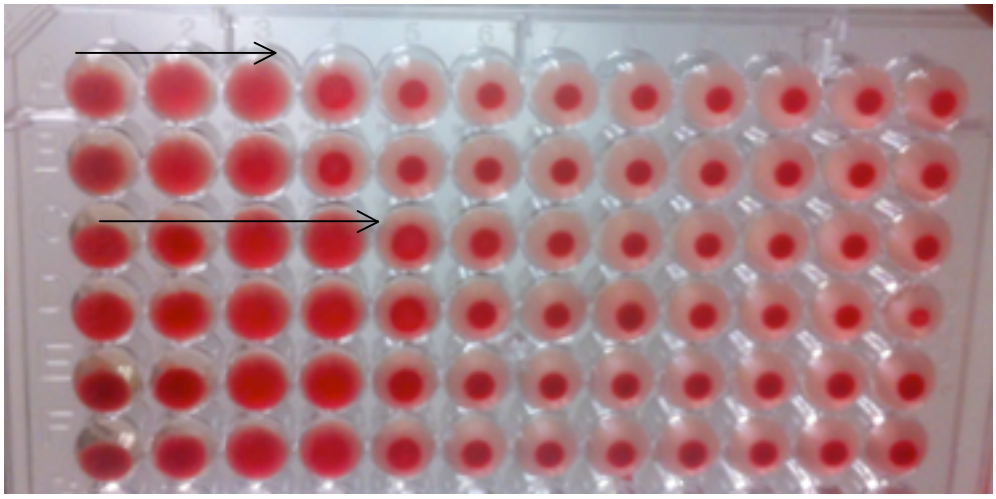

Fig. 2: Microtiter plate showing results of an agglutination assay. The positive results appear as red carpet layer in wells numbered 1 to 3, rows A and B, and 1 to 4, rows C to F (indicated by arrows), and negative results as a red button (as seen through wells 4 to 12, rows A and B and 5 to 12, rows C to F)

Different factors i.e. blood group specificity; pH and seasonal variation are known to affect lectin activities (Naeem *et al*., 2007; Muramoto *et al*., 1991; Nsimba-Lubaki and Peumans, 1986; Costa *et al.,* 2010). In this study, how these factors affect *Q. fusiformis* lectin activity were investigated. Results of the study will aid in the characterization of *Q. fusiformis* lectin.

Blood group specificity study of *Q. fusiformis* lectin

The blood group specificity of *Q. fusiformis* lectin activity was investigated in four blood groups (horse, human, rabbit and sheep). The *Q. fusiformis* leaf lectin crude extracts were assayed using blood from different organisms. The extracts of *Q. fusiformis* agglutinated to all blood groups tested, making *Q. fusiformis* a non-blood group specific lectin. Lectins can be blood group specific; some lectins may agglutinate to red cells of one type of blood and not others, while a non-blood group specific lectin demonstrates agglutination to different blood from different organisms. Lectin specific activity was expressed as titer over milligrams of protein (SA). For analysis, lectin specific activity was transformed to natural logarithm of specific activity (ln SA). ANOVA results reported a difference among the four blood groups tested, which was subjected to a Bonferroni test using a Type I error rate (α of 5%) for mean comparisons to determine significant differences (Table 1, Figure 1). The Bonferroni test showed a significant difference in the ln specific activity values of *Q. fusiformis* lectin between sheep blood (ln SA=8.30) and the three blood groups (horse, ln SA= 6.29), human (ln SA= 7.21) and rabbit (ln SA= 7.02). As shown in Figure 3, ln specific activity using sheep blood showed a significantly higher lectin activity as compared to the other three blood groups. However, lectin ln specific activities of *Q. fusiformis* using horse, human and rabbit blood groups were not significantly different from each other.

Specificity is influenced by the limited number of contacts with carbohydrates and depth of the sugar binding sites (Drickamer, 1995). In addition, any modification or substitution to a binding site can influence binding specificity (Drickamer and Taylor, 2002). Both structural and molecular features of lectin and carbohydrate must be taken into account. For example, lectin will vary in amino acid sequences, as well as in three-dimensional conformation structures.

Table 1: Determination of blood group specificity of *Q. fusiformis* crude extract lectin, summary of mean results recorded

Blood group	Titer[a]	Protein Content(mg/mL)[b]	HA[c]	SA (titer/mg)[d]	ln SA[e]
Horse	6.08	0.743	304	959.59	6.29[f]
Human	18.16	0.743	908	2094.11	7.21[f]
Rabbit	15.2	0.743	760	2542.26	7.02[f]
Sheep	49.44	0.743	2472	10654.62	8.3[g]

[a] Titer is the reciprocal of the lowest dilution that was positive for lectin activity.
[b] Protein content was determined using Bradford assay.
[c] Hemagglutination Activity (HA), is titer multiplied with sample volume (50 uL).
[d] SA, Specific Activity is HA divided by the protein content.
[e] ln Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from Bonferroni test using a type I error rate of 5%. The assays were done in five replications.
[f] Blood groups horse, human and rabbit show no significant difference from each other.
[g] Sheep blood group is significantly different from horse, human and rabbit.

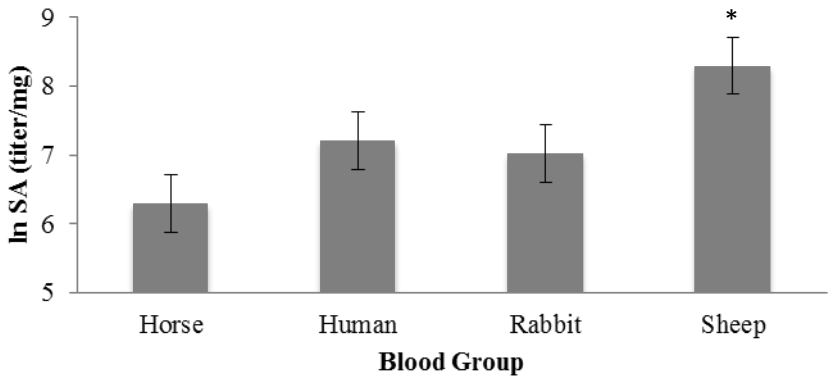

Fig. 3: Blood group specificity of *Q. fusiformis* lectin based on ln SA of mean comparisons analyzed with a Bonferroni test using a type I error rate of 5%. *Significant ($P<0.05$) compared to horse, human and rabbit blood groups

Carbohydrate structures may be simple or complex, varying in configuration and distinguished arrangement of hydroxyl groups (Drickamer, 1997). Drickamer (1997) classified monosaccharide affinities of lectin-carbohydrate binding into two groups. The first group is glucose, mannose, and N- acetylglucosamine because of a shared common arrangement of equatorial 3- and 4- hydroxyl groups. The second group consists of galactose and N-

acetylgalactosamine, which share the presence of an axial 4-hydroxyl group (Drickamer, 1997). Examples of reported monosaccharide specificities reported include leaves from the orchid Twayblade, *Listera ovata* which expresses exclusive specificity towards mannose (Van Damme *et al*., 1987), *Dolichos biflorus* seeds with galactose-specificity (Wu and Sugii, 1991), while potato tubers express specificity towards N-acetylglucosamine (Allen and Neuberger, 1973) and *Ulex europeus* seeds express fucose-specificity (Pereira *et al*., 1978). Oligosaccharides are exceptional molecules because they adopt a variety of shapes and are naturally flexible. Oligosaccharide-lectin interactions, especially with multi-branched oligosaccharides, exhibit a significant increase in lectin binding reactivity compared with linear counterparts (Wu and Sugii, 1991).

The observed differences in *Q. fusiformis* lectin activity with different blood groups may be due to differences in carbohydrate-lectin binding interactions which can be attributed to differences in carbohydrates presented on the cell surfaces of the different blood groups. The carbohydrates on the erythrocyte cellular surfaces are distinguishable among the four different blood groups (Khan *et al*., 2008). The reported monosaccharide determinants in the four blood groups are, fucose in horse (Wu *et al*., 2009), galactose in human and (Wu *et al*., 2009; Kusui and Takasaki, 1998; Shibuya *et al*., 1988) and mannose in rabbit erythrocytes (Yagi *et al*., 2002). In human erythrocytes there are seven monosaccharide components that vary in combination, contrasting in serial arrangements from one another and different in the nature of chemical linkages among the sugars galactose, mannose, fucose, glucose, acetylglucosamine and N-acetylneuraminic (sialic) acid (Bird, 1975). Horse, human and rabbit red blood cells may contain carbohydrate components on the cellular surface binding sites that are relatively less recognized by the *Q. fusiformis* lectin binding site. On the other hand, the carbohydrates found on the

cellular surface of sheep red blood cells may contain carbohydrate units in a structure and position more specific and with higher affinity for the binding of *Q. fusiformis* lectin, subsequently increasing sheep erythrocyte agglutination.

Although galactose is a monosaccharide determinant in both human and sheep erythrocytes it can be assumed that the sugar arrangements and nature of chemical linkages of the sugars in sheep erythrocytes may be different in comparison to human erythrocytes. Perhaps, how the sugars are arranged and linked in sheep's blood is recognized with higher affinity by the *Q. fusiformis* lectin binding site. This lectin could have an extending site or multivalency. The term extending site is the ability to recognize additional sugars linked to primary determinant (Weis and Drickamer, 1996). Moreover, lectin characteristics such as, multivalency may determine cross-linking interaction in binding recognition. Spatial distribution of multivalency among lectin structures may produce a higher level of specificity (Loris, 2002; Sacchettini *et al*., 2001).

It has been reported that plant lectins differ in molecular structures and specificities (Peumans and Van Damme, 1995). *Quercus fusiformis* lectin activity is similar to other plant lectins that are also non-blood group specific. Konozy *et al*. (2002) investigated blood-group specificity from seeds of *Erythrina speciosa* against a wide range of blood groups. Its lectin activity was examined in the human blood ABO system and animal blood groups, rabbit, mouse, sheep and horse. *Erythrina speciosa* lectin was characterized as a non-blood group specific. Likewise, the blood group specificity for leaf lectin in *Kalanochoe crenata* was characterized as a non-blood group specific lectin, agglutinating to the different types of human blood red cells of the ABO system (Adenike and Eretan, 2004). *Artocarpus incisa* seeds were also examined in a wide range of blood groups including human ABO system and animal blood groups, cow, goat,

rabbit, pig and sheep. *Artocarpus incisa* seed lectin resulted in non-blood group specificity in humans ABO system, while rabbit blood group activity was not different from human ABO and the other four blood groups were significantly different from human and rabbit agglutination (Moreira *et al*., 1998). Similarly, *Bryopsis plumosa* lectin from a green marine alga agglutinated sheep and horse erythrocytes (Han *et al*., 2010). The blood group specificity of *Q. fusiformis* lectin activity suggests *Q. fusiformis* lectin is non-blood group specific with higher specificity directed towards sheep erythrocytes. The present study is the first to report the non-blood group specificity of the *Q. fusiformis* lectin.

Galactose serves as the monosaccharide determinant in sheep red blood cells (Wu *et al*., 2009; Kusai and Takasaki, 1998). Thus the demonstrated higher specificity of *Q. fusiformis* lectin towards sheep red blood cells leads to the possibility that *Q. fusiformis* lectin may bind to galactose with higher affinity. In this regard the *Q. fusiformis* lectin may be isolated using a galactose affinity column chromatography. For example, Korean mistletoe leaf lectin, a galactose specific lectin was purified with the use of D-galactose affinity matrix column (Park *et al*., 1997).

Effect of pH on *Q. fusiformis* lectin activity

The effect of pH on lectin activity was determined. Lectin-carbohydrate interactions depend on the lectin's binding specificity towards the carbohydrates, which is associated with the lectin's three-dimensional structure. Lectins' three-dimensional structures are maintained through hydrogen bonds, van der Waals forces, ionic interactions and disulfide linkages. Any disruptions to these interactions change the physical properties that then affect biological activities of the lectin. Hydrogen bonding and van der Waals contacts are the dominant forces that stabilize lectin-carbohydrate interactions (Quiocho, 1986). Likewise, lectin binding sites

contains amino acids with dissociable functional groups that contribute to positive and negative charges as well as hydroxyl residues. Any pH change is associated with a change in the ionic state of functional groups. These changes affect ionic interactions on the surface of molecules that influence binding forces of a lectin and thus affecting its activity (Adenike and Eretan, 2004). The lectin stability and activity may decrease when pH values are more acidic or basic (Utarabhand and Akkayanont, 1995).

Crude extracts of *Q. fusiformis* lectin were prepared and incubated in wide range of pH values for 24 hours with stirring at 4°C (cold room temperature). Each pH level was assayed with the four blood groups. Figure 4, shows the effect of pH on lectin activity for *Q. fusiformis*. A Bonferroni test using a Type I error rate (α of 5%) to compare mean natural logarithm-transformed specific activity (ln SA) across pH levels (5.2, 6.2, 7.2 and 8.2) indicated no significant differences, while that for pH 9.2 was significantly lower than for the other four levels (Table 2). In other words, findings indicated that *Q. fusiformis* lectin is stable at a pH range 5.2 to 8.2. Different pH conditions were found to have profound effects on the tertiary and quaternary structure of proteins and can perturb protein conformational stability (Ugwu and Atpe, 2004). Lectins have different optimum pH to maintain their stability. Literature reported that pH stabilities of plant lectins among species vary. For example, *Morus rubra* (Sureshkumar and Priya, 2012) and *Ipomoea asarifolia* (Salles *et al*., 2011) leaf lectin crude extracts were stable at pH 7.5, while the Korean mistletoe lectin crude extract was stable at pH 8.0 (Park *et al*., 1997).

The pH stability of *Q. fusiformis* lectin activity at a wide range of pH is quite similar to other leaf lectins. Adenike and Eretan (2004) investigated the pH stability of *Kalanochoe crenata* leaf lectin at pH values 2 to 12. *Kalanochoe crenata* leaf lectin activity was stable from 2 to 7.5,

and the activity was lost at a pH higher than 9.2. The effect of pH on lectin activity was studied in the leaves of *Chorchorus olitours* also in a broad range of pH values from pH 2.0 to 10.5. *Chorchorus olitours* exhibited a high agglutination activity from pH 7.2 to 8.0 with a dramatic decrease of lectin activity below acidic conditions of pH 6.5 and above basic pH of 9.0 *(*Khan *et al*., 2008). The study demonstrated the lectin to be pH sensitive. Utarabhand and Akkayanont (1995) report when lectin pH values are modified, either for an acidic or basic pH conditions lectin stability and activity decreases. *Quercus fusiformis* lectin pH results are in agreement with Utarabhand and Akkayanont (1995). Furthermore, it was reported that different buffer compositions can also alter protein-surfactant binding characteristics and alter protein conformation, chemically modifying covalent structure and affecting protein stability (Ugwu and Apte, 2004).

Effect of seasonal variation on *Q. fusiformis* lectin activity

Since the productivity of plants (such as biomolecule synthesis) is easily changed by seasonal variation, that affects environmental factors such as soil properties, moisture and pH is also examined to determine if any of these factors affect lectin activity in *Q. fusiformis*. Seasonal variation contributes to notable changes in water, light and nutrient availability. Several resource-based hypotheses propose that defense compounds of plants are synthesized based on the external availability of resources and internal trade-offs. For example, carbon/nutrient balance (CNB) predicts that environmental variation induces change of the nutrient availability, needed for plant growth, will also directly affect quantitative changes in carbon and nitrogen productions (Bryant *et al*., 1987). On the other hand, the growth/differentiation balance (GDB) of plant defense hypothesis is a physiological tradeoff between growth and differentiation (Herms and Mattson, 1992). The tradeoff between growth and defense is growth and defense is

Table 2: Effect of pH on *Q. fusiformis* crude extract lectin, summary mean values recorded

pH	Titer[a]	Protein Content (ug/uL)[b]	HA[c]	SA(titer/mg)[d]	ln SA[e]
5.2	18.6	0.532	930	2912.27	7.33[f]
6.2	19.8	0.697	990	3053.03	7.25[f]
7.2	36.1	0.856	1805	8147.53	7.73[f]
8.2	24.5	0.78	1225	4944.42	7.32[f]
9.2	12.5	0.847	607	1255.98	6.39[g]

[a] Titer is the reciprocal of the lowest dilution that was positive for lectin activity.
[b] Protein content was determined using Bradford assay.
[c] Hemagglutination Activity (HA), is titer multiplied with sample volume (50 uL).
[d] SA, Specific Activity is HA divided by the protein content.
[e] ln Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from Bonferroni test using a type I error rate of 5%. The assays were done in five replications.
[f] pH levels not significant difference from each other, lectin activity was stable.
[g] pH level significantly different from 5.2-9.2.

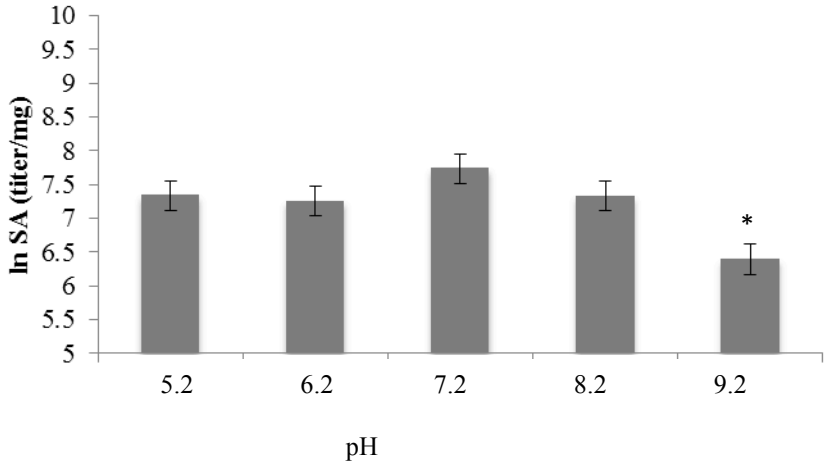

Fig. 4: The effect of pH on *Q. fusiformis* lectin activity based on mean comparisons of ln SA analyzed with a Bonferroni test using a type I error rate of 5%. *Significant, ($P<0.05$) compared to pH range of 5.2-8.2

due to secondary metabolites limited to dividing cells and diverting resources of production to new leaves, thus creating a dilemma in plants. They must grow fast enough to complete and maintain plant defenses in the presences of predations and pathogens (Herms and Mattson, 1992). Agglutination assays of *Q. fusiformis* crude extracts were done and compared. Lectin activities were measured in the summer, fall, and winter.

Summer season consisted of hot temperatures with an average of 104°F, with mostly clear skies, and long days. In the fall season, hot temperatures were reduced to an average temperature of 93°F, with partly cloudy skies and with the most rain due to hurricane season. The winter season consisted of an average temperature of 50°F, with cloudy skies and short days. Two fall seasons were recorded, fall 2011 and fall 2012. Also, two winter seasons were observed in 2011 and 2012 while only one summer season was observed.

Samples were collected from three trees in Fall 2011 and 2012. Also, samples collected were never after rain events due to water saturating soil and increasing water content. The mean ln SA (specific activity) was 7.6. Samples were collected from three trees in the winter season in 2011 and 2012. The mean ln SA for winter was 7.8. Samples were collected from three trees in the summer season for 2012 in which, the ln SA value was 8.2. Results of the correlation analysis determined that there was no significant correlation in mean ln SA for *Q. fusiformis* lectin during the different seasons, fall, winter and summer (Figure 5). Studies have reported other lectin sources that display a seasonal variation in lectin activity. Moreover, lectins in Elderberry (*Sambucus nigra*) and Black locust (*Robinia pseudoacacia*) bark demonstrated a seasonal variation in content since accumulation of lectin is believed to occur in autumn and lessen during the spring (Nsimba-Lubaki and Peumans, 1986). On the contrary, lectins in cultivated red alga *(Kappaphycus alvarezii*) concurred with seasonal variation, lectin content varied with environmental characteristics such as heavy rain, solar radiation and low seawater temperatures (Hung *et al*., 2009). However, Nsimba-Lubaki and Peumans (1986) hypothesize that lectin fluctuation found in both Elderberry and Black locust barks contributed seasonal variation thereby resembling the behavior of protein storage organelles. This is in agreement with Peumans and Van Damme (1995) reiterating that plant lectin is a heterogeneous group of

proteins. Due to their wide occurrence in the plant kingdom and their distribution in different tissues, many plant lectins mimic the behavior of plant storage proteins. Seed lectin and non-seed lectin (vegetative tissue) express storage protein behavior but should not be classified as storage proteins but in close association, which is also true for some leaves and stems of plant species (Peumans and Van Damme, 1995). In the barks of Elderberry and Black Locust, lectin accumulates during summer to winter and depletes in the spring season. However, this was attributed to leaf maturity which could influence lectin activity. Costa *et al*. (2010) studied *Phthirusa pyrilfolia* leaf lectin activity and reported mistletoe to be sensitive to seasonality and require sunny weather to promote high lectin expression due to mistletoe's slow rate of photosynthesis. Our studies have shown that *Q. fusiformis* lectin from oak tress grown at Laredo, Texas was not affected by seasonal variations.

Effect of soil moisture and soil pH on *Q. fusiformis* lectin activity

Plant growth is a response to soil conditions in terms of water and nutrient availability provide to the roots (Passioura, 2002). Soil varies in conditions such as physical (dryness), chemical (salinity) and biological (infection) attributes (Passioura, 2002). Soil moisture is a component of soil hardiness that affects root growth. Soil properties influence the growth and activity of organisms and plant growth and development (Angers and Caron, 1998). Also, soil conditions vary in regards to acidity or alkalinity and indicate soil nutrient availability. Acidic soil conditions tend to have fewer macronutrients available and alkaline soil conditions have fewer micronutrients available. To date, the literature with regards to soil the properties of moisture and pH affecting lectin activity are scarce. The lectin activity of common beans (*Phaseolus vulgaris*) was examined in three different soil types in a semiarid region. The result of this study reports differences in lectin concentration and suggests environmental factors

Table 3: Effect of seasonal variation of *Q. fusiformis* crude extract lectin, summary of average results recorded in seasons

Seasons	Titer[a]	Protein Content[b]	HA[c]	SA (titer/mg)[d]	ln SA[e]
Fall	5	0.15683	200	2540.06	7.6676
Winter	9	0.165167	450	2792.97	7.8715
Summer	4	0.112667	200	3930.9	8.2296

[a] Titer is the reciprocal of the lowest dilution that was positive for lectin activity.
[b] Protein content was determined using Bradford assay.
[c] Hemagglutination Activity (HA), is titer multiplied with sample volume (50 uL).
[d] SA, Specific Activity is HA divided by the protein content.
[e] ln Specific activity (SA), is the natural logarithm of specific activity. Values of ln SA are mean values from One-way ANOVA, t-test analysis. The assays were done in three replications.

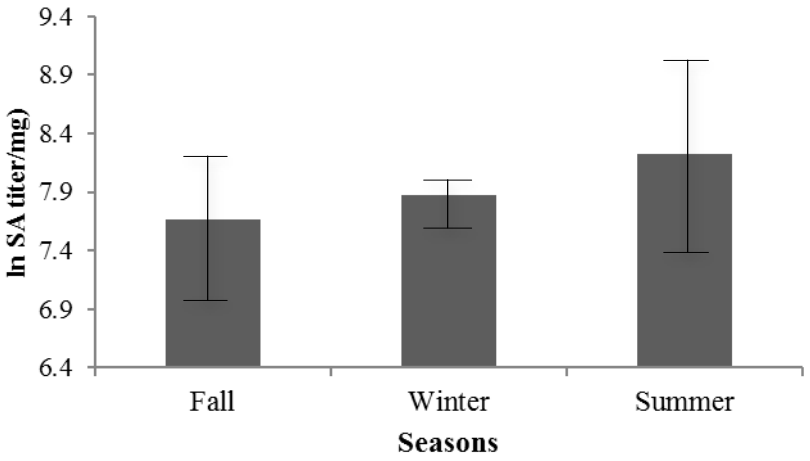

Fig. 5: Seasonal variation results from correlation analysis of lectin mean ln SA

to contribute to lectin difference (Gonzalez de Mejía *et al*., 2003).

Quercus fusiformis tree samples were from the TAMIU campus. The location of each tree was exposed to similar abiotic environmental factors such as being close to parking lots and had the same exposure to solar radiation. Soils encompassing the samples were collected and examined at three separate depths: 0-10 centimeters, 10-20 centimeters and 20-30 centimeters. However, *Q. fusiformis* roots are at least 50 centimeters deep (Simpson, 1999; Tull and Miller, 1999), thus depth focused on was the 20-30 centimeter depth, which was the next best estimation

for soil analysis. The average soil pH reading for 20-30 centimeter depth in fall was pH 8.36 and for winter pH 8.31. On the other hand, the average moisture loss for the 20-30 centimeter depth in fall was 3% and for winter 5.6%. The 20-30 centimeter soil moisture and pH were subjected to a correlation analysis with level of significance for a two-tailed test at level 0.01 as shown in Table 4. Soil moisture and pH reflect the chemical status of soil conditions for the *Q. fusiformis*. Most trees species will grow in a broad range of soil pH values (Williston and LaFayette, 1978). Laredo soil is usually alkaline throughout (USDA, 1985). Since the pH results of soil are basic indicating there may be some nutrients unavailable to *Q. fusiformis*, the production and expression of lectin may be affected. On the other hand, *Q. fusiformis* is a drought-tolerant tree and only requires water every three to four weeks. Low soil moisture content may reflect the drought conditions occurring throughout Texas. Analysis of the results for soil moisture and soil pH of the 20-30 centimeter depth showed no significant correlation with ln SA for *Q. fusiformis* lectin.

Determination of the antifungal activity of *Q. fusiformis* extracts

There are two physiological proposed roles for plant lectins; the first is in plant defense mechanisms and the second, symbiotic nitrogen-fixing bacteria association (Sharon and Lis, 2004). Plant lectins have been investigated for their resistance to insects (Vandenborre *et al*., 2011), bacteria (Ayouba *et al*., 1994) and fungi (Peumans and Van Damme, 1995). The study *Q. fusiformis* lectin is relevant since new compounds and especially plant lectins with antifungal, activity are of high interest due to rising fungal resistance to common fungicides. Fungi are widely distributed in nature and are persistent pathogens in different plant species. Fungi have been one of the main causes for crop and cultivated plant losses (Vu and Huynh, 1994). There are only a few plant lectins reported to have antifungal activities. The fungal cell walls are thick

Table 4: Correlation analysis for the effect of soil moisture and soil pH on lectin activity of *Q. fusiformis*

		ln SA	SA	soil moisture 20-30cm	soil pH 20-30cm
ln SA	Correlation	1	0.972	0.454**	0.479**
	Sig. (2-tailed)		0.000	0.089	0.071
	N	15	15	15	15
SA	Correlation	0.972	1	0.432**	0.442**
	Sig. (2-tailed)	0.000		0.108	0.099
	N	15	15	15	15
soil moisture 20-30cm	Correlation	0.454**	0.432**	1	0.844
	Sig. (2-tailed)	0.089	0.108		0.000
	N	15	15	15	15
soil pH 20-30cm	Correlation	0.479**	0.442**	0.844	1
	Sig. (2-tailed)	0.071	0.099	0.000	
	N	15	15	15	15

**. Correlation is significant at the level 0.01 (2-tailed)

and rigid with chitin as the main cellular component composed of monosaccharide units of N-acetylglucosamine (Peumans and Van Damme, 1995). Due to the rigid composition of the fungal cell wall, lectin has no direct affect in altering the structure or permeability of fungal cell walls (Peumans and Van Damme, 1995; De Souza Cândido *et al*., 2011). Hence, plant lectin antifungal properties would refer to the potential inhibition of fungal growth but not a fungicidal activity. Since oak leaves were observed to be often attacked by fungi, the potential antifungal activity was examined in *Q. fusiformis* lectin.

The assay for *Q. fusiformis* lectin was tested against *Aspergillus niger* and *Rhizophus stolonifer*. These two fungi are common pathogens in fruits and vegetables and cause rot disease (Yildirim *et al*., 2010; Sharma, 2012). Potato dextrose plates with post mycelial growth were exposed to paper discs containing 20 µL aliquot solutions of freeze-dried crude extracts of *Q.*

fusiformis. Freeze-dried crude extracts of 125 µg/µl, 250 µg/µl and 500 µg/µl concentrations did not show any antifungal activity against *A. niger* and *R. stolonifer* (Figure 7). The 500 µg/µl was the highest concentration that can be prepared from the freezed-dried crude extracts; concentrations higher than 500 µg/µL resulted in non-homogenous solutions. Similarly, knife bean (*Canavalia gladiata*) lectin, was devoid of antifungal activity when tested against three fungi *Botrytis cinerea, Myocospharella arachidcola* and *Fusarium oxysporum* (Wong and Ng, 2005). In contrast, plant mistletoe (*Phthirusa pyrfolia)* lectin was tested against eleven fungi (A*spergilus niger*, *A. flavus, A. fumigatus, Rhizopus arrhizue, Paeciloyces variottie, Fusarium moniliforme, F. laterituium, Candida albicans, C. burneses, C. tropicalis, C. parapsilosis, Saccharomyces cerevisiae* and *Rhizoctnia solani*) and demonstrated antifungal activity against two fungi *F. lateritium and R. solani* (Costa *et al*., 2010). Red kidney bean *(Phaseolus vulgaris)* lectin reported by Ye *et al*. (2001), expressed antifungal activity towards three fungal species, *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus*. Ye *et al*. (2001) attributed red kidney bean activity to lectin's structural resemblance to chitinases. Chitinases are known to negatively affect hyphal growth and ultimately cause cell wall disruption, liberate chitin oligosaccharides from cell wall and cause leakage of cytoplasm (Benhamou *et al*., 1993).

Purification of *Q. fusiformis* lectin

The partial purification of *Q. fusiformis* lectin consisted of crude extraction and a one-step chromatography technique. The crude extraction of lectin from *Q. fusiformis* was achieved by homogenization in Trizma-HCl, pH 9.2(0.15 M NaCl) buffer and stirred at speed 3 (VWR® Dyla-Dual™ Hot Plate Stirrer, Product No. 12620-970) at 4°C for one hour.

Partial purification of *Q. fusiformis* crude extract was carried out by a one-step column chromatography technique using the ion-exchange chromatography column. The weak

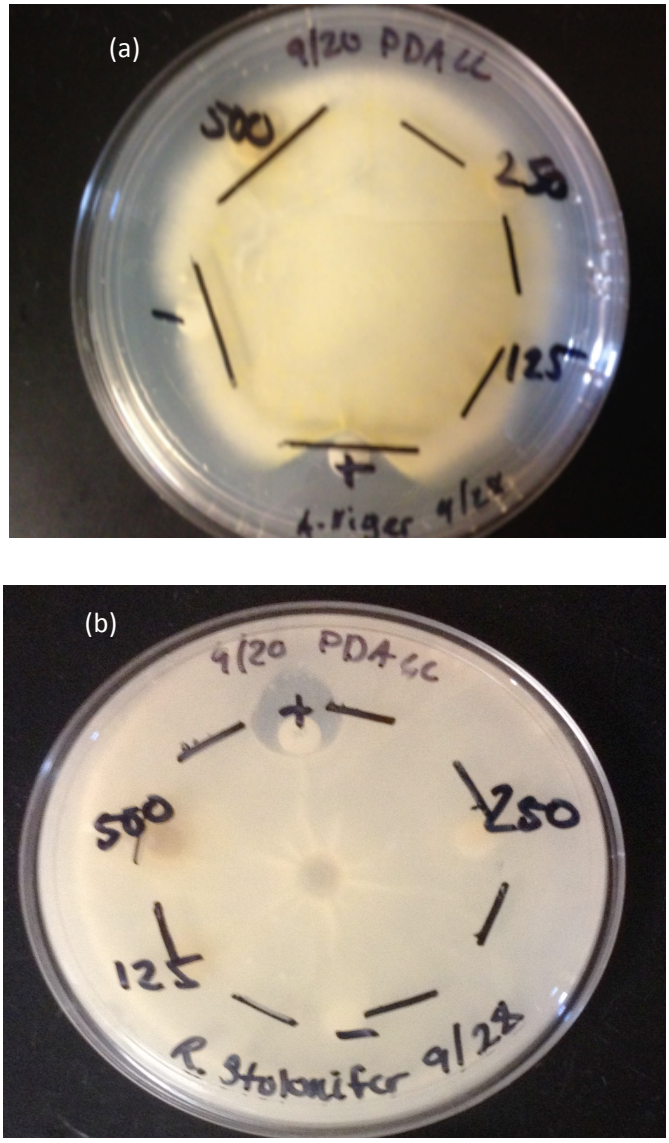

Fig. 6 (a-b): *Q. fusiformis* lectin devoid of antifungal activity against (a) *A. niger* and (b) *R. stolonifer.* The negative control was DMSO and positive (+) control was the antifungal agent, nystatin

anionic DEAE-cellulose column (Pall Life Sciences AcroSep™, Product No. 20067-C001) consists of a matrix with positive charges that bound negatively charged lectins. The crude extract was prepared using basic buffer 0.01 M Trizma-HCl buffer pH 9.4. In preliminary studies, crude extracts of 0.01 M PBS, pH 7.4; 0.01 M Trizma-HCL, pH 9.4 were prepared and applied to a CMC column (matrix with negative charges) and DEAE column, respectively. CMC

column chromatography using extracts at pH 7.4 did not bind any protein as evidence by the presence of only one peak that corresponded to the unbound proteins (chromatograph not shown).

Crude extracts of *Q. fusiformis* at pH 9.2 ran through the DEAE column chromatography resulted in the binding of the lectin. The chromatography profile showed three peaks, peaks are labeled with abbreviation letter D to indicate DEAE. As shown in Figure 9, the *Q. fusiformis* crude extract resulted in the separation of proteins that corresponded to 3 peaks. The first peak (D1), the major peak, contained unbound proteins. The second peak (D2) and third peak (D3) corresponded to bound proteins that were eluted from the DEAE column using half stepwise salt concentration gradient 0.5 and 1.0M, respectively. The purification procedure by Kuku *et al*. (2009) also involved anionic-exchange chromatography by applying *C. annum* lectin crude extract through DEAE column and three peaks were also obtained. The two cultivars of Chinese black soybeans were purified through a series of chromatographic steps, with the first chromatographic step using anion-exchange chromatography through Q Sepharose column with three adsorbed fractions obtained (Lin *et al*., 2008).

Quercus fusiformis DEAE fractions, D1, D2 and D3 were tested for agglutination activities. These fractions did not exhibit any agglutinating activity. The absence of lectin activity in DEAE fractions may be for two reasons. First, fractions may not contain enough lectin concentration to have an activity detectable by the sensitivity of the agglutination assay due to dilution effect of column chromatography. Secondly, the high salt concentration used to elute proteins in peaks D2 and D3 may indicate an effect on conformational structure and stability of the lectin. Thus, fractions from each peak were pooled, dialyzed and lyophilized. Dialyzed samples were measured for pH with litmus paper and demonstrated a neutral pH. Lectin

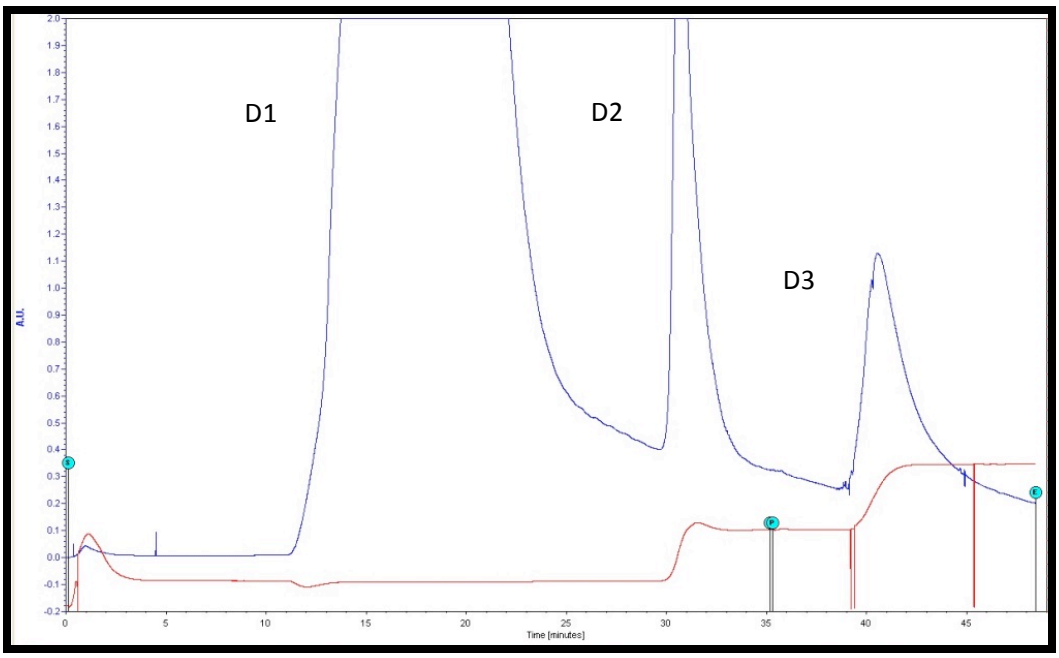

Fig. 7: Purification of *Q. fusiformis* lectin by DEAE ion-exchange column chromatography

Table 5: Lectin activity of the dialyzed and lyophilized fractions from D1, D2 and D3[a]

Peak	Titer	HA
D1	144	7200
D2	20	1000
D3	10	500

[a]Results were averaged of two replications.

activity is affected when pH conditions are modified for basic or acidic conditions (Utrabhand and Akkayanont, 1995). This is true for *Q. fusiformis* lectin activity, and provides support from the effect of pH results when lectin activity decrease at pH level 9.2. The lyophilized samples were diluted with 200 µl of millipore water and subjected to agglutination assays to detect the presence of lectin activity. Table 5 shows results of the agglutination assays. Results showed that all peaks contained lectin activity. The first peak, D1 contained all unbound proteins i.e. unbound lectin, thus D1 was not subjected to purity determination. Between D2 and D3, D2 was

chosen for purity determination owing to the relatively higher protein concentration as well as higher lectin activity observed in peak 2, D2. The purity of sample D2 was assessed using high performance liquid chromatography (HPLC).

The binding of *Q. fusiformis* lectin to the DEAE column could be attributed to the lectin amino acid composition. It can be assumed that *Q. fusiformis* lectin has a predominance of negatively charged aspartic acid and/or glutamic acid residues. These amino acids would give the lectin a net negative charge at the basic pH due to the R-carboxylic groups (COO^-) present. Thus, the negatively charged *Q. fusiformis* lectins bind to the positive charged matrix of DEAE. For example, red marine alga (*Vidalia obtusiloba* C.) lectin was purified through two chromatographic steps, with its first step being an ion exchange DEAE-cellulose column. This lectin was reported to be rich in aspartic acid, glutamic acid and leucine (Melo *et al*., 2004).

Determination of *Q. fusiformis* purity via HPLC

Analytical assessment of purity was done using High Performance Liquid Chromatography (HPLC). Based on the analytical assessment of D2, Q. *fusiformis* lectin is either only partially purified or D2 contains at least three isolectins since 3 peaks were obtained after HPLC. Isolectins are defined as closely related lectins difficult to separate since they have a similar amino acid composition. The purification and characterization of two lectins from *Araucaria brasiliensis* seeds showed an HPLC elution profile with one symmetrical peak each for lectin I and lectin II (Datta *et al*., 1991). On the other hand, *Arisaema flavum* purified using affinity chromatography was reported to be purified to homogeneity owing to one single peak after HPLC (Singh *et al*., 2004).

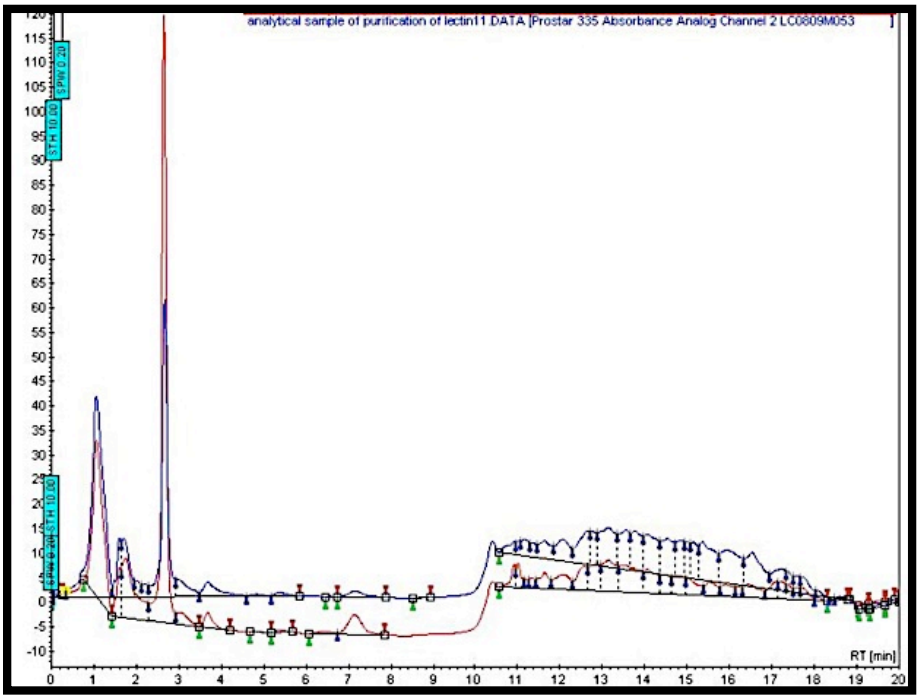

Fig. 8: HPLC elution profile of peak 2, D2 from DEAE ion exchange column chromatography of *Q. fusiformis* extract showed three peaks

CONCLUSIONS AND RECOMMENDATIONS

Lectin in *Quercus fusiformis* was non-blood group specific, lectin agglutinated to the four different blood groups. However, *Q. fusiformis* lectin had a higher affinity for sheep blood which was significantly different from horse, human and rabbit blood groups. Also, *Q. fusiformis* lectin was stable within a pH range of pH 5.2 to 8.2, with optimal activity at 7.2 and a significant decrease in activity at pH 9.2. The pH sensitivity of *Q. fusiformis* lectin indicates the three-dimensional conformational structure and its binding sites are disrupted by ionic interactions occurring in different buffers and pH values. Seasonal variation and environmental factors have been reported to affect lectin activity. Seasonal variation was examined and no significant difference was found in lectin activity in *Q. fusiformis.* Environmental factors examined in this study focused on soil properties such as soil moisture and pH had no significant effect on lectin activity in *Q. fusiformis.*

The one step ion-exchange column chromatography technique with DEAE column resulted in three adsorbed peaks. The latter peaks contained lectin activity. HPLC results indicated a partially purified lectin. Lectin is hypothesized to play a role in plant defense and exhibit antifungal properties. Crude extracts from *Q. fusiformis* were examined for antifungal activity for potential inhibition and was devoid of fungal activity in the two fungi tested, *Aspergillus niger* and *Rhizopus stolonifer.* Results of the present study provide a characterization and the partial purification of *Q. fusiformis* lectin. Future efforts are needed that include upscale purification and further characterization of the lectin under study. The sugar inhibition test may reveal sugar specificity of *Q. fusiformis* lectin. Highly concentrated lectin and purified lectin may be examined for other biological activities including antifungal activities. In addition, determination of antifungal activities against other fungal species is recommended. The DEAE

isolate can be further purified using a galactose affinity column owing to *Q. fusiformis* observed affinity to sheep's blood.

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