ANTIMICROBIAL ACTIVITIES OF SAPONIN-RICH GUAR MEAL EXTRACT

A Dissertation

by

SHERIF MOHAMED HASSAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Aubrey L. Cartwright
Committee Members,	Christopher A. Bailey
	James A. Byrd
	Michael E. Hume
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ABSTRACT

Antimicrobial Activities of Saponin-Rich Guar Meal Extract. (May 2008)

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Chair of Advisory Committee: Dr. Aubrey Lee Cartwright

Three saponin-rich extracts (20, 60, 100% methanol), four 100% methanol sub-fractions and seven independently acquired fractions (A-G) from guar meal, *Cyamopsis tetragonoloba* L. (syn. *C. psoraloides*), were evaluated for antimicrobial and hemolytic activities. These activities were compared against quillaja bark (*Quillaja saponaria*), yucca (*Yucca schidigera*), and soybean (*Glycine max*) saponins in 96-well plates using eight concentrations (0.01 to 1.0 and 0.1 to 12.5 mg extract/mL). Initial guar meal butanol extract was 4.8 ± 0.6% of the weight of original material dry matter (DM). Butanol extract was purified by preparative reverse-phase C-18 chromatography. Two fractions eluted with 20, and one each with 60, and 100% methanol with average yields of 1.72 ± 0.47, 0.88 ± 0.16, 0.91 ± 0.16 and 1.55 ± 0.15% of DM, respectively. Further purification of the 100% methanol fraction using normal-phase silica gel preparatory high pressure liquid chromatography eluted 4 peaks at 16, 39, 44 and 46 min. Only the 100% methanol fraction, its 16 min peak, F and G fractions, and quillaja saponin, exhibited both hemolytic and antibacterial activities against *Staphylococcus aureus*, *Salmonella* Typhimurium and *E. coli*, but 20 and 60% methanol fractions stimulated *Lactobacillus spp.* growth. Guar meal (0 or 5%) was added to diets fed to chicks from 1

to 21 days of age. Chicks fed both diets were unchallenged or challenged with 5 x 10^3 *Eimeria tenella* sporulated oocysts at 10 days. Guar meal diets reduced oocysts shed per gram of feces, body weight, and feed efficiency. Adding 2.5% guar meal, 1% guar gum, or 0.125% saponin-rich guar meal extract to diets fed to chicks to 21 days of age showed that guar meal increased the cfu concentrations of digesta more than controls following a challenge with 10^7 cfu of *Clostridium perfringens* at 14 days. Body weights of chicks fed guar meal and saponin-rich extract were significantly lower than control body weights at 21 days of age, whereas the weekly feed to gain ratio of chicks fed saponin-rich extract was higher than controls. Guar meal reduced severity of *Eimeria tenella* infection and guar saponin-rich extract exhibited antimicrobial activity against several common poultry pathogens.

DEDICATION

My love and gratitude is expressed to my parents, my wife and my entire family for their continued encouragement, support, and sacrifices throughout my educational pursuits and daily life.

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Most importantly, I express my love and gratitude to my parents, my wife, my children and the entire family for their patience, love, continual encouragement, support, sacrifices and prayers throughout my educational pursuits and daily life.

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CHAPTER I

INTRODUCTION

Antibiotics have been used extensively in animal feed to stimulate growth rate and to inhibit growth of intestinal pathogens. However, continued feeding of antibiotics at sub-therapeutic levels has created concerns about the development of drug resistant bacteria, reduced ability to cure bacterial infections in humans, and antibiotic residue in food (Jensen, 1998). Antibiotics used in animal production are no longer desirable because of concerns about increasing antibiotic resistant pathogenic microorganisms in both veterinary and human medicine (Nawaz et al., 2000; Bertolatti et al., 2003; Wallace, 2004).

Increased awareness of potential problems associated with the use of antibiotics as feed additives stimulated research efforts to identify alternatives to their use. Some antibiotic alternative products such as probiotics, prebiotics (Murry et al., 2004), organic acids (Van Immerseel et al., 2006) and medicinal herbs (Wink, 1999; Gordon and David, 2001; Du and Hu, 2004; Arab et al., 2006) have been used to treat or prevent diseases. Original candidate chemical structures for many pharmaceutical compounds used to promote human health originated from chemicals found in plant extracts (Du and Hu, 2004; Arab et al., 2006). Among these plant compounds are saponins from quillaja and yucca which have several beneficial effects on animal health (Sen et al, 1998b; Avato et

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This dissertation follows the style of Poultry Science.

al., 2006). Saponins are glycoside compounds distributed in many plants (Price et al., 1987; Mahato et al., 1988; Shimoyamada et al., 1990), lower marine animals and some bacteria (Riguera, 1997). The chemical structures of saponins consist of a fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) or steroid (C-27) attached with one or more sugar side chains (glycone) at different carbon sites of the aglycone. Saponins have many biological properties, among them are hemolytic (Fukuda et al., 1985; Khalil and El-Adawy, 1994; Woldemichael and Wink, 2001), antibacterial (Mahato et al., 1982; Sen et al., 1998b; Avato et al., 2006; Hassan et al., 2007) and antiprotozoal activities (Mshvildadze et al., 2000).

Guar (*Gyamopsis tetragonoloba*) is saponin-rich plant that contains 5-13% saponin by weight of dry matter (Curl et al., 1986; Hassan et al., 2007). Guar is a drought tolerant annual legume mainly grown for the guar gum (galactomannan polysaccharide) that has many industrial and food processing applications. Guar meal is a combination of hull and germ fractions that is produced as a by-product of guar gum manufacture. In addition, guar meal contains 33-47.5% crude protein (Ambegaokar et al., 1969) that makes it potentially useful as an ingredient for poultry feed (Lee et al., 2003a, b; 2005). Guar meal also contains 13-18% residual galactomannan gum (Bakshi et al., 1964; Lee et al., 2004). Guar meal has characteristics of a useful feeding ingredient that could also have antimicrobial activity because of galactomannan and saponin content. Guar seed contains both saponin and galactomannan polysaccharides which could be of use as natural antibacterial compounds (Hussain and Cheeke, 1995; Tanaka et al., 1996; Sen et al., 1998b; Van Nevel et al., 2005).

The most predominant pathogenic bacterial and protozoal groups in chickens include *Staphylococcus aureus* (Huys et al., 2005a, b), *Escherichia coli* (Turtura et al., 1990), *Salmonella* Typhimurium (Foley et al., 2007), *Clostridium perfringens* (Van Immerseel et al., 2004), and *Eimeria tenella* (Shirley, 1986; Williams, 1998, 1999; 2005). The treatment and prevention of bacterial and protozoal diseases are costly to the poultry industry. Shane and Van der Sluis (2002) estimated that the cost of control methods of all infectious disease in poultry in the USA could amount to almost $3 billion per annum. Research has evaluated neither the hemolytic nor antimicrobial activities of saponin-rich guar meal extracts. Therefore, the objectives of this research were to isolate saponin-rich extract from guar meal to determine in vitro antibacterial activities against relevant gram-positive bacteria (*Staphylococcus aureus, Clostridium perfringens* and *Lactobacillus spp*.) and gram-negative bacteria (*Salmonella* Typhimurium and *Escherichia coli*) in a dose dependent manner. Also, anticoccidial and anti-*Colstridium perfringens* activities were evaluated in vivo by adding guar meal, purified guar gum or saponin-rich extracts to feed of chicks challenged with the disease organisms.

CHAPTER II

LITERATURE REVIEW

Pathogenic Bacterial and Coccidial Diseases

Bacterial and protozoal diseases cause significant economic loses in the poultry industry. Shane and Van der Sluis (2002) estimated that the cost of control methods for infectious disease in poultry in the USA could amount to almost $3 billion per annum. Smith (1965) reported that the first organisms to colonize the alimentary tract of most animals, including the chick, are *Escherichia coli*, *Salmonella spp*., *Clostridium perfringens*, and *Streptococci*. These bacteria are also major food borne pathogens associated with processed poultry that cause severe illness and even death in humans (Tauxe, 1991).

This dissertation is focused on the most predominant pathogenic bacterial and protozoal groups that include *Staphylococcus aureus* (Huys et al., 2005a, b), *Salmonella* Typhimurium (Foley et al., 2007), *Escherichia coli* (Turtura et al., 1990), *Clostridium perfringens* (Van Immerseel et al., 2004) *Eimeria tenella* (Shirley, 1986; Williams, 1998; 1999; 2005) and one beneficial bacterium (*Lactobacillus spp.*).

Staphylococcus aureus

Staphylococcus aureus is a gram-positive facultative anaerobe and opportunistic pathogen. Enterotoxin-producing *Staphylococcus aureus* is the most common cause of food-borne human illness throughout the world (Do Carmo et al., 2004; Le Loir et al.,

2003). *Staphylococcus aureus* produces hyaluronidase that destroys tissues and also produces coagulase enzyme that causes clot formation (Ryan and Ray, 2004) and can cause bumblefoot in chickens. *Staphylococcus aureus* infections can be spread through contact with pus from an infected wound, skin-to-skin contact and contact with objects such as towels, sheets, clothing, or athletic equipment. *Staphylococcus aureus* frequently resides on the skin, in the nose, and in bone, joint and endovascular wound infections. Approximately 20–37.2% of the general human population is *Staphylococcus* carriers (Kluytmans et al., 1997; Heyman, 2004).

Staphylococcus aureus causes disease problems such as septicaemia and skeletal infections in commercial broiler chickens (Jordan and Pattison, 1996). Bacterial chondronecrosis of the proximal end of the femur and/or tibiotarsus, associated mainly with *Staphylococcus aureus*, was identified as the predominant cause of lameness in two commercial broiler chicken flocks (McNamee et al., 1998). It is a significant cause of avian disease and may thus contaminate carcasses processed for food (Mead and Dodd, 1990).

While *Staphylococc*i commonly occur on the skin and nasopharynx of healthy poultry (Mead and Dodd, 1990), it is primarily *Staphylococcus aureus* which can survive, colonize, and persist at various processing stages in commercial poultry processing plants due to the expression of various key properties, including adhesion (Chaffey and Waites, 1987; Mead et al., 1995) and chlorine resistance (Dodd et al., 1988; Huys et al., 2005a, b). The poultry processing plants are favorable environments for the survival and transmission of various commensal, spoilage, and potentially

pathogenic bacteria in the human food chain (Huys et al., 2005a, b). *Staphylococci* are one of the most predominant bacterial species encountered during poultry slaughter and processing. They have been recovered from air samples (Ellerbroek, 1997), neck skin of chicken carcasses (Geornaras et al., 1995; Olivier et al., 1996), and equipment and machinery surfaces (Geornaras et al., 1995; Huys et al., 2005a, b).

The foods that most frequently cause *Staphylococcus aureus* food poisoning are red meat and poultry and their products (Balaban and Rasooly, 2000; Genigeorgis, 1989; Kitai et al., 2005; Wieneke et al., 1993). In the UK, 53% of the staphylococcal food poisoning cases reported between 1969 and 1990 were due to meat products and meat-based dishes, and 22% of the cases were due to poultry and poultry-based meals (Le Loir et al., 2003; Wieneke et al., 1993).

Salmonella spp.

Salmonella is a gram-negative facultative rod-shaped bacterium in the same family as *Escherichia coli* (*Enterobacteriaceae* or enteric bacteria) that live in the intestinal tracts of warm and cold blooded animals. Since Daniel E. Salmon discovered the first strain of *Salmonella* (*Salmonella choleraesuis*) in 1885 (Salmon and Smith, 1886), the number of strains technically termed serotypes or serovars of *Salmonella* known to cause salmonellosis has increased to over 2,300. Currently, genus *Salmonella* infections rank second to genus *Campylobacter* as the major causes of food-borne infections. Salmonellosis is the most frequent disease caused by *Salmonella* in the USA, and is the second most common food-borne illness in humans worldwide (Feng, 1992;

Aabo et al., 1995; Tietjen and Fung, 1995; Whyte et al., 2002). The predominance of this genus has caused increased public awareness and consumer apprehension when purchasing poultry products.

Salmonella can be found in virtually every part of the world and carried by an extremely wide variety of hosts including humans and other mammals, birds, reptiles, and insects (Austin and Wilkins, 1998; Gast, 1997; Kusters et al., 1993; Sato et al., 1999). *Salmonella* are responsible for a variety of acute and chronic diseases in both poultry and humans. In humans, *Salmonella* are the cause of two diseases called salmonellosis: 1) enteric fever (*Salmonella* Typhimurium is the strain that causes typhoid fever) resulting from bacterial invasion of the bloodstream, and 2) acute gastroenteritis, resulting from a food-borne infection/intoxication. ***Salmonella spp.*** infections continue to plague the poultry industry and cause substantial losses in productivity. Infected poultry products are among important sources for food-borne outbreaks in humans. ***Salmonella spp.***, among them *Salmonella* Typhimurium, have long been major causative agents of food-borne infection and has been a concern of the poultry industry. ***Salmonella spp.*** has been focus of numerous scientific investigations aimed at eliminating the bacterium (Slutsker et al., 1998).

Although the *Salmonella* serotypes most significant in human disease are not pathogenic to poultry. These serotypes remain important as a contaminant of poultry as food. A 1991 survey of poultry in the Netherlands reported that fecal samples from 94% of meat-type broilers, 86% of 406 layer houses and 47% of egg-type layer flocks were *Salmonella* positive (Edel, 1994; Van de Giessen et al., 1991). Similarly, in 1994, 53%

of flocks tested from either fecal or egg belt sampling in Canada were positive for *Salmonella spp*. (Poppe et al., 1991). In studies of pooled cecal samples from egg-layers in southern USA detected *Salmonella* in 100% of the flocks (Waltman et al., 1992; Ebel et al., 1992).

According to the Centers for Disease Control and Prevention, *Salmonella* affects about 1.4 million people each year with about 20,000 hospitalizations and 500 deaths annually in the USA (Mead et al., 1999). In 1996, the United States Department of Agriculture, Economic Research Service estimated that the total costs for medical care and lost productivity, resulting from food-borne *Salmonella* infections of humans was between 0.6–3.5 billion dollars annually (USDA, 1996). The USA annual salmonellosis burden was estimated to be about 1.5 million cases (including over 580 deaths), 95% of these cases were attributed to food-borne infection (Mead et al., 1999). Other costs associated with *Salmonella* include various direct expenses producers face as a consequence of *Salmonella* infection in their flocks. Moreover, *Salmonella* contamination of food products can significantly reduce consumer demand and affect producer profits (Myint, 2004).

The prevalence of *Salmonella spp.* in poultry has prompted implementation of antibiotic regimens designed to eliminate *Salmonella* with antibiotics often being administered in the feed as growth promoters (Moellering, 1998; Cruchaga et al., 2001; Kramer et al., 2001). These practices have evolved bacterial strains exhibiting antibiotic resistance to all known antibiotics (Rubin and Weinstein, 1977; Levy et al., 1987; Aarestrup, 1999; Witte et al., 2000). The growing global concern over antibiotic

resistance and the stigma of *Salmonella spp.* associated with poultry has resulted in increased research efforts designed to eliminate *Salmonella spp.* infections.

Escherichia coli

Escherichia coli bacteria were discovered in the human colon in 1885 by German bacteriologist Dr. Theodor Escherich who demonstrated that certain strains of bacteria were responsible for infant diarrhea and gastroenteritis (Feng et al., 2002). *Escherichia coli* are gram-negative, facultative bacteria which grow either aerobically or anerobically (Abrams, 1983; Mason and Richardson, 1981; Furth and Guiot, 1989; Neidhardt et al., 1990), and are commonly found in the lower intestine of warm-blooded animals such as chickens, deer, sheep, and pigs (Vogt and Dippold, 2005).

Enteritis caused by *Escherichia coli* (colibacilliosis) is an important disease in the poultry industry because of increased mortality and decreased performance (Barnes et al., 2003). *Escherichia coli* is able to produce Shiga-like toxins, or verotoxins that inhibit protein synthesis in eukaryotic cells, and play a role in hemorrhagic colitis and hemolytic uremic syndrome by causing damage to endothelial cells in the kidneys, pancreas, brain, and other organs (Griffin and Tauxe, 1991).

Escherichia coli were first recognized as a food-borne pathogen associated with consumption of hamburgers from a fast food chain restaurant (Riley et al., 1983). In the ten years that followed, approximately thirty *Escherichia coli* outbreaks were recorded in the USA. The Centers for Disease Control and Prevention (CDC) based on a 1999 estimated 73,000 cases of *Escherichia coli* infections occur annually in the USA (CDC,

2006). Every year, 2,100 Americans are hospitalized, and 61 people die as a direct result of *Escherichia coli* infections.

The majority of food-borne illness outbreaks associated with *Escherichia coli* have involved ground beef, unpasteurized apple and orange juice (Cody et al., 1999), unpasteurized milk, alfalfa sprouts, lettuce, and contaminated water (Friedman et al., 1999). A study on the prevalence of *Escherichia coli* in livestock at 29 county and 3 large state agricultural fairs in the USA found that *Escherichia coli* could be isolated from 13.8% of beef cattle, 5.9% of dairy cattle, 3.6% of pigs, 5.2% of sheep, and 2.8% of goats (Keen et al., 2003). The estimated annual cost of *Escherichia coli* illnesses are $405 million (Frenzen et al., 2005). Costs contributing to this estimate included $370 million for premature deaths, $30 million for medical care, and $5 million for lost productivity.

Escherichia coli resistance against many known antibiotics has been reported (Gupta et al., 2001; Pitout et al., 2005; Garau et al., 1999; Johnson et al., 2006). One suspected source of drug-resistant *Escherichia coli* in humans is the use of antimicrobial drugs in agriculture food production (Linton, 1977; Jones and Schaffner, 2005; Collignon and Angulo, 2006). Supporting this hypothesis is the high prevalence of antimicrobial drug–resistant *Escherichia coli* in retail meat products, especially poultry (Johnson et al., 2003; Johnson et al., 2005a, b; Schroeder et al., 2003).

Clostridium perfringens

Clostridium perfringens is a gram-positive, spore-forming anaerobic bacterium that is ubiquitous in the environment, including water, soil, workers clothing and boots (Willis, 1977). *Clostridium perfringens* is often found in relatively small numbers (< 10^4 cfu) in the gastrointestinal tract (GIT) of most bird species (Johansson and Sarles, 1948; Shapiro and Sarles, 1949; Gazdzinski and Julian, 1992; Branton et al., 1997; Asaoka et al., 2004) as a normal inhabitant of gut microflora of many animals (Smith, 1965). *Clostridium perfringens* may be found in the crop, gizzard, small intestine and ceca. The presence of *Clostridium perfringens* does not lead directly to necrotic enteritis (NE) disease. Although 75-95% of birds are colonized by *Clostridium perfringens*, only a small proportion of these ever show symptoms of NE disease.

Necrotic enteritis in poultry is associated with α-and β-toxin producing *Clostridium perfringens* strains type A or C (Van Immerseel et al., 2004). All strain types (A-E) of *Clostridium perfringens* bacterium contain the α-toxin (McDonel, 1986; Songer, 1996; Petit et al., 2001). Clostridial β-toxin induces hemorrhagic necrosis of the intestinal mucosa (Baba et al., 1992). Necrotic enteritis affects a variety of bird species (Cowen et al., 1987) such as chickens (Broussard et al., 1986), turkeys (Gazdzinski and Julian, 1992; Droual et al., 1995) and quail (Berkhoff, 1985).

Necrotic enteritis was first reported in a flock of 6-7 week-old cockerels in England (Parish, 1961). Chickens from 2-24 weeks of age are affected (Schwartz, 1988; Ficken, 1991) with the most common incidence at 2.5-3 weeks of age between July to October (Long, 1973; Ross, 1999). Many factors are attributed to NE induction such as

diet composition (Smith, 1965; Nairn and Bamford, 1967), high levels of fish meal (Johnson and Pinedo, 1971; Truscott and Al-Sheikhly, 1977) or wheat (Branton et al., 1987; 1997).

The first indications that birds may be suffering from NE are huddling, ruffled feathers, depression in growth rate, and diarrhea followed by an increase in mortality rates (Porter, 1998). Necrotic enteritis symptoms can be divided into sub-clinical and clinical categories. Sub-clinical symptoms include depression in appetite, feed efficiency and growth rate (Kaldhusdal and Hofshagen, 1992; Porter, 1998; Lovland and Kaldhusdal, 2001). Clinical symptoms include inappatence, diarrhea and severe necrosis of the intestinal tract that ultimately can lead to mortality (Ficken and Wages, 1997).

Upon examination of the gastrointestinal tract and associated organs of affected birds, gross lesions usually are found in the jejunum and ileum. The proximate small intestine and ceca show less evidence of lesions, but may be distended by gas and bloody fluids (Frame and Bickford, 1986). The intestinal mucosa may be covered with a layer of fibrino-necrotic material (Porter, 1998).

Necrotic enteritis has high economic and animal welfare costs. The disease has become increasingly prevalent in the European Union due to factors such as the use of diets often containing wheat, and the removal of antibiotic growth promoters and animal by-products from diet formulations. Necrotic enteritis costs producers as much as 5 cents per bird in the USA (Van der Sluis, 2000). Estimates of NE incidence vary from 1-40% of commercial broiler flocks in North America and the European Union (Kaldhusdal and

Skjerve, 1996; Kaldhusdal and Lovland, 2000; Annett et al., 2002). New preventatives and treatments for NE are needed.

Eimeria tenella

Avian coccidiosis (genus *Eimeria*) is a disease with an estimated $800 million worldwide cost for treatments employed to prevent the disease in poultry industry (Williams, 1998). The economic impact is much higher due to its negative effects of coccidiosis on the productive performance of poultry. Although, seven species of *Eimeria* (*Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria necatrix, Eimeria praecox,* and *Eimeria tenella*) infect chickens, *Eimeria tenella* is the most prevalent species found in the USA poultry operations. *Eimeria tenella* causes high incidence and high mortality (50-70%) coccidiosis in poultry leading to significant economic losses (Williams, 1999). This protozoan parasite exists and is spread as a highly resistant oocyst in litter (Shirley, 1986). Currently, antiprotozoal chemical feed additives are the most widely used means of controlling *Eimeria* in chickens (Pogonka et al., 2003). While effective for avian coccidiosis, continuous use and misuse of anticoccidial drugs have led to the emergence of drug resistant strains (Long, 1982). Drug resistance in coccidial populations is a constant threat to the continued success of prophylactic chemotherapy.

Lactobacillus spp.

Lactobacillus spp. are gram-positive, non-spore-forming, rods that produce lactic acid (Ayres et al., 1980). The genus *Lactobacillus* is the largest of the lactic acid producing bacteria or LAB genera. *Lactobacilli* are the most acid-tolerant of the LAB which produce antimicrobial concentrations of lactic acid. They can grow well under both microaerophic to anaerobic conditions (Neidhardt et al., 1990). They produce lactic acid as the major end product during fermentation of carbohydrates (Brock et al., 1994). Bacteriocins, which inhibit growth of other bacteria, are proteinaceous compounds produced by a large and diverse group of *Lactobacillus spp.* Bacteriocins inhibit potential food-borne pathogens (Barefoot and Klaenhammer, 1983). The antibacterial effects of *Lactobacillus spp.* are due to their production of not only bacteriocins, but also organic acids. Blom and Mortvedt (1991) reported that acetic acid produced by *Lactobacillus spp.* has inhibitory effects on yeasts, molds, and other bacteria. A mixture of lactic and acetic acids suppresses the growth rate of *Salmonella* Typhimurium more than either acid alone (Rubin, 1978). *Lactobacillus spp*. are the most prominent members of mammalian microflora found in the oral cavity, gastrointestinal tract, and vagina of animals (Sharpe, 1981; Kandler and Weiss, 1986).

Dietary incorporation of probiotics and organic acids has gained attention as a viable growth promoting option in the poultry industry. Probiotics are defined as viable microorganisms, that when ingested, exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance and reducing or controlling food-borne pathogens such as *Salmonella, Escherichia coli, Staphylococcus aureus* infections

thereby improving performance (Hentges, 1992). The genus *Lactobacillus* is classified as a probiotic (Lee and Salminen, 1995). Probiotic supplements are associated with a number of health claims that include diarrhea prevention (Yolken et al., 1994), antitumorigenic effects (Takano et al., 1985), and cholesterol reduction (Tahri et al., 1995).

Antibiotic Resistance Is a Growing Problem

Antibiotics had their origins in 1928 when Alexander Flemming discovered that a substance produced by the fungus *Penicillium notatum* (penicillin) effectively inhibited the growth of *Staphylococcus aureus* on agar plates. Approximately 13 years later, penicillin was purified and successfully used as a treatment (Witte, 2000). Following the development of penicillin, numerous other antibiotics such as streptomycin in 1943 and cephalosporins in 1953 were developed (Witte, 2000). Today, approximately 5000 different antibiotics are known and about 100 of these are in use (Witte, 2000).

Penicillin was introduced into the USA in the 1940's and was recognized as a wonder drug due to its ability to eliminate pathogenic diseases. Antibiotics are defined as natural (produced by microorganisms as a defense mechanism) or synthetic compounds that are capable of inhibiting proliferation or destroying microorganisms (Levy, 1998).

An increase in resistance to antibiotics by many microbes has been observed within the last decade to the extent that microbes resistant to every known antibiotic have been identified. The problem of antibiotic resistance is not a new. The first reported

treatment failure occurred in 1936 when normally reliable sulphonamides failed to treat gonorrhea infections (Witte, 2000). Three years later, *Staphylococcus aureus* had developed penicillinase, capable of neutralizing the antibiotic (Neu, 1992b).

Additionally, the use of benzyl penicillin to treat staphylococcal infections became increasingly unsuccessful during the 1940's (Witte, 2000). *Staphylococcus aureus* was the first pathogen resistant to multiple antibiotics such as penicillin, streptomycin, oxytetracycline, and erythromycin in the 1950's (Witte, 2000). This incident led to development of the first semisynthetic anti-s*taphylococcal* antibiotic, methicillin (Witte, 2000). However, a methicillin-resistant *Staphylococcus aureus* had been isolated even before methicillin was in the market (Barber, 1961; Witte, 2000). By the 1980's, methicillin resistant *Staphylococcus aureus* (MRSA) was a problem in the USA (Lyon and Skurray, 1987; Chambers, 1988; Neu, 1992b). By 1992, approximately 95% of *Staphylococcus aureus* were resistant to not only to penicillin, but also to ampicillin and antipseudomonas penicillins (Lyon and Skurray, 1987; Neu, 1992b). Over 2000 serovars of *Salmonella spp.* possess some degree of antibiotic resistance (O'Brien, 1987; Tenover, 1991; Neu, 1992a, b; Low et al., 1997; Glynn et al., 1998). In the Netherlands the observed increase in *Salmonella spp*. resistance to tetracycline between 1959 and 1974 resulted in the ban of tetracycline use as a growth promoter in animal feed (Aarestrup et al., 2000).

Bacteria resist antibiotics through four basic mechanisms. The antibiotic can be destroyed by altering antibiotic structure. This mechanism is illustrated by the natural resistance of gram-negative bacteria to vancomycin and nafcillin, as well as, the

resistance of *Enterococci* bacteria (*Clostridia, Staphylococci, Lactobacilli spp.*) to aminoglycosides (Murray, 1991). The second mechanism utilizes an enzyme to alter a single amino acid in enzymes that affect the target site's affinity for some antibiotics as for β-lactamases (Neu, 1992b). The third mechanism acts by expelling the antibiotic from the bacterium such as β-lactamases, aminoglcosides, and tetracyclines (Neu, 1992b). The fourth mode of action decreases cell wall permeability to the antibiotic (Murray, 1991; Livermore, 2000; Witte, 2000).

Increased incidence of antibiotic resistance arises from a number of practices. Chief among them are continuous feeding, inappropriate application, feeding of low doses for extended periods, and overuse of antibiotics in the agricultural and medical sectors (Aarestrup, 1999). Antibiotics are used as growth promoters in livestock and poultry production, as well as, in crop production and fish farming (Levy 1998; Aarestrup, 1999; Witte et al., 2000). Estimates indicate that approximately 40% of the 50 million pounds of antibiotics produced annually are administered to animals (Levy, 1998). Some of this represents legitimate use to treat infections; however, a large proportion is used for growth promotion in the swine and poultry industries. In 1954, two million pounds of antibiotics were produced while today more than 50 million pounds are produced (Levy, 1998). In 1988, 80,000 tons of antibiotics were used for agricultural purposes in the USA (Khatachatourians, 1998; Ungemach, 2000).

Antibiotic resistance negatively impacts the economy in the USA and other countries as a result of prolonged illness, increased mortality, and more frequent and prolonged hospitalization (Holmberg et al., 1987; Williams, 2000). Treatment costs also

increase as the use of newer more expensive antibiotics are required to replace older antibiotics. Unfortunately, these newer more expensive antibiotics are often unavailable in some countries (Levy, 1998; Williams, 2000).

Awareness of potential problems associated with antimicrobial resistance evolving from the use of antibiotics as feed additives has focused research efforts to identify alternatives for controlling infections and increasing performance in animal production (Vicente et al., 2007). Recent experience indicates that development of new drugs may be a losing battle since bacteria develop resistance at faster rates than new drug discovery.

Researchers worldwide are working to develop antibiotic alternative products such as probiotics, prebiotics (Drake et al., 2003; Patterson and Burkholder, 2003; Murry et al., 2004; Higgins et al., 2005b; 2007; Tellez et al., 2006), organic acids (Van Immerseel et al., 2006) and medicinal herbs (Wink, 1999; Gordon and David, 2001; Du and Hu, 2004; Arab et al., 2006). Non-traditional chemicals (Moore et al., 2006) and bacteriophages (Higgins et al., 2005a; Vicente et al., 2005) have been used in the last decade to treat and prevent pathogenic diseases, and improve poultry performance. Original candidate chemical structures for many pharmaceutical compounds used to promote human health originated from chemicals found in plant extracts (Du and Hu, 2004; Arab et al., 2006). Among these plant compounds are saponins from quillaja, and yucca which have several beneficial effects such as antibacterial and antiprotozoal activities (Sen et al, 1998b; Avato et al., 2006).

Saponin as an Antibiotic Alternative

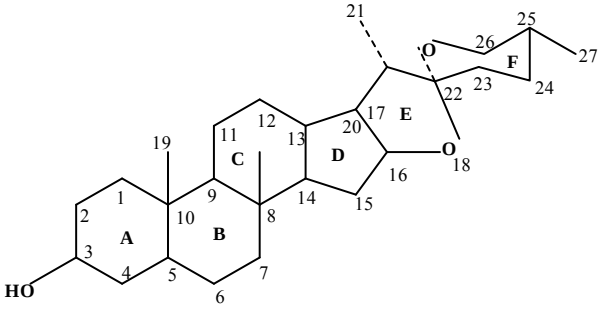
Characteristics of Saponins

Saponins acquired their name from the soapwort plant (Saponaria root) which was used as soap. Saponins are generally identified by their bitter taste, throat irritation, form foam in aqueous solutions (Mahato et al., 1988; Cheeke, 1998), fish toxicity (Mahato et al., 1988) and ability to lyse erythrocytes. However, an example of exceptions is, ginsenoside saponins do not lyse erythrocytes (Gogelein and Huby, 1984; McManus et al., 1993; Takechi and Tanaka, 1995a, b). Some saponins even have been used as flavor enhancers and sweeteners in foods and cigarettes. For example, the flavor enhancer, licorice root extract is rich in the saponin glycyrrhizin, and saponins from the roots of *Glycyrrhiza glabra* and leaves of *Abrus precatorius* (Oleszek et al., 1992; Tanaka et al., 1996) are 941-fold as sweet as sucrose and 60 times sweeter than cane sugar (Mizutani, 1994).

Chemical Nature of Saponins

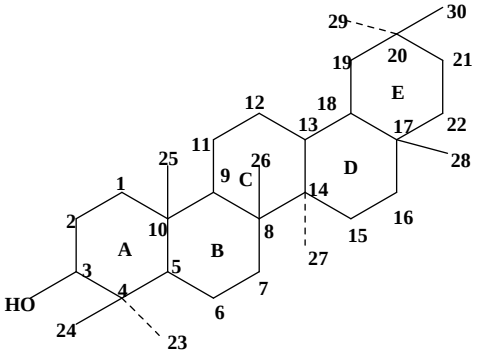
Saponins are synthesized by a common metabolic pathway starting from acetyl coenzyme A. Mevalonic acid and then squalene are the intermediary products for both triterpenoidal and steroidal saponins. In general, synthesis of cholesterol, other steroids, and saponins proceed through a common synthetic pathway.

Saponins are glycoside compounds (Tanaka et al., 2000; Oleszek et al., 2001a, b) whose chemical structures (Figure 1) are composed of a fat-soluble nucleus called the



Steroid Neutral Class

Steroid Alkaloid Class



Triterpenoid Class

Figure 1. Skeletal types of aglycone found in the three principal classes of saponin.

aglycone that is a either triterpenoid (C-30), or neutral or alkaloid steroids (C-27) (Price et al., 1987; Hostettmann et al., 1991). One or more sugar side chains called glycones can be linked through ether and ester linkages to the aglycone nucleus at glycosylation sites (Cheeke, 1998). Triterpenoid saponins naturally occur as saponin or free aglycone forms, while steroid saponins occur only as saponins and never in the free aglycone form. The molecular weights of saponins range from 1000 to 1500 Daltons (Dorsaz and Hostettmann, 1986).

Saponin Distribution

Saponins are distributed in both wild and cultivated plants (Price et al., 1987; Mahato et al., 1988; Shimoyamada et al., 1990), in lower marine animals and in some bacteria (Riguera, 1997), but are uncommon in higher animals (Hashimoto, 1979). Triterpenoid saponins are widely distributed in the plant kingdom and have been identified in over 500 plant species such as soybean, alfalfa, quillaja, peas, tea, spinach, sugar beet, quinoa, liquorices, sunflower, horse chestnut, ginseng, and guar (Basu and Rastogi, 1967; Fenwick et al., 1991). Steroid saponins occur predominantly in 85 species of the genera *Agave*, *Discorea* and *Yucca* and 56 other genera such as tomato, asparagus, ginseng, and oats (Fenwick et al., 1991). In legumes, saponins are associated with protein and therefore are concentrated in protein-rich fractions (Fenwick and Oakenfull, 1981; Curl et al., 1986). Two major commercial sources of saponins are yucca (*Yucca schidigera*) and quillaja (Q*uillaja saponaria*). Yucca is grown in the arid Mexican desert and southwestern USA, and quillaja is a tree grown in arid areas of Chile.

Types and numbers of saponin differ in their distribution among plants according to many factors such as the part, species, and age of the plant. More than one kind of saponin may occur in the same species. Alfalfa saponins containing zanhic acid aglycone (trisdesmoside alfalfa saponin containing three sugar side chains attached to aglycone) and its γ-lacton (lucernic acid or glucuronic acid) are found in leaves but not in roots (Massiot et al., 1988). Medicagenic acid aglycones saponin (bisdesmoside alfalfa saponin containing two sugar side chains attached to aglycone) is found in the roots and is absent in the plant leaves (Oleszek, 1996) and hederagnin saponins (monodesmoside alfalfa saponin containing one sugar side chain attached to aglycone) are identified in both roots and leaves of alfalfa (Jurzysta, 1982; Tava et al., 1993). Soybeans have at least four different saponins (Berhow et al., 2006). The saponins in the mature soybean are divided into group A and group B soyasaponins on the basis of their aglycone structures. Group B soyasaponins appear to exist in the intact plant tissue as a conjugate of 2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) at the 22 hydroxy position (Shiraiwa et al., 1991a; Kuduo et al., 1993). Group A soyasaponins are bisdesmosidic with alternate sugar compositions in both sets of oligosaccharides attached to the aglycone at the 3- and 21-hydroxyl positions (Shiraiwa et al., 1991b). Group A saponins are found only in soybean hypocotyls, while group B saponins are widely distributed in legume seeds in both hypocotyls (germ) and cotyledons (Shiraiwa et al., 1991a).

Saponin Concentrations

Saponin concentrations differ among plants as a function of plant species, plant variety (Shiraiwa et al., 1991a, b), cuttings of the same plant (Ng et al., 1994), plant part, degree of maturity, growing environment (sunlight intensity, rain, disease and insect attack, etc.), agronomic factors (climate and soil), cultivation year, location grown and season.

As a percentage of dry matter, guar meal contains 5-13% saponin (Curl et al., 1986; Hassan et al., 2007) while soybean seed (*Glycine max*, Leguminosae Merrill) contains 0.5-6.5% (Ireland et al., 1986; Berhow et al., 2006), yucca contains 8-12% (Kaneda et al., 1987), quillaja contains 8-10% (Hostettmann and Marston, 1995), alfalfa contains 0.5-9.5% (Nowacka and Oleszek, 1992; Oleszek, 1996), licorice root contains more than 3% (Tanaka et al., 1996), fenugreek seed contains 5-6% (Sauvaire et al., 1996) and the aerial part of *Medicago arborea* contains 1.9-3.4% saponins of dry matter (Nowacka and Oleszek, 1994).

Saponin concentration tends to be lower in the outer parts than the inner parts of the oat kernel, but is the converse in quinoa seeds (Onning and Asp, 1995). Alfalfa roots contain 2.41% while alfalfa leaves contain 1.53% of dry matter as saponin (Gestetner et al., 1971). Alfafla (*Medicago sativa* L.) roots and leaves contain saponins ranging from 2.6-3.8 and 0.3-2.4% of dry matter, respectively. Quinoa saponins are 0.9% and 2.3% of dry matter in the whole seed and the bran, respectively (Ruales and Nair, 1993).

Saponin concentration of a plant during germination is higher than in a mature plant of the same species (Pedersen, 1975). For example, alfalfa saponin concentration in

sprouts increases from 2 mg/g at the beginning of germination (soyasaponin I) up to 6-8 mg/g (0.6-0.8% of dry matter) at 8-16 d of age. Although saponin contents increase with sprouting in some plants such as soybean, lucerne, mung beans, and peas, they decrease in others plants such as moth beans (Yoshiki et al., 1998).

Planting season affects saponin content. Alfalfa saponin content is lower in spring and fall than content in midsummer (Burda et al., 1994). Zanhic acid saponin (aglycone is zanhic acid) is the highest late in the season when the level of medicagenic acid saponin (the precursor of zanhic acid) drops dramatically (Oleszek, 1996). Hederagenin saponins in alfalfa leaves ranged from 0.1-0.4% of the total aglycosides (Jurzysta, 1982) or 0.03 mg/g of dry matter (Tava et al., 1993) versus 0.82-1.32 mg/g in alfalfa root. Also, concentrations of saponin types A, B and E of alfalfa leaves are 10-15.5, 15-30, and 1%, respectively (Jurzysta, 1982).

Extraction procedure also plays a role in reported net saponin concentrations. For example, oat saponin content ranges between 0.011 and 0.029% of dry matter as determined by high pressure liquid chromatography (HPLC) (Onning et al., 1993). However, avenacoside A oat saponin was 0.04% of dry matter (Tschesche et al., 1969) and avenacoside B was 1% of dry matter (Fenwick and Oakenfull, 1983) as determined by thin layer chromatography (TLC).

Saponin Extractions

Although most saponins lack a chromophore, which makes the task of isolating saponins from plant material complicated, many methods are available for saponin

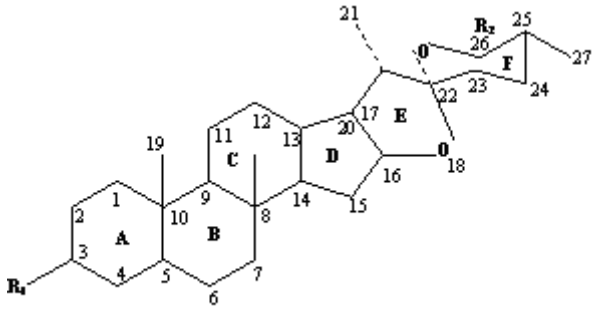
extraction from different plants (Marston et al., 2000; Muir et al., 2000; Schopke, 2000; Berhow et al., 2006). Saponins have been extracted by refluxing in several organic solvent systems such as pure methanol (Shany et al., 1970; Gestetner et al., 1971; Massiot et al., 1988; 1991; Oleszek et al., 1992), different ratios of methanol:water, 4:1 (v:v) (Massiot et al., 1991), 3:7 (v:v) (Oleszek et al., 1992), ethanol:water, 4:1 (v:v) (Levy et al., 1989; Oleszek et al., 1990; Oleszek and Bialy, 2006), or even pure water (Massiot et al., 1991). After the evaporation of initial solvent, the extract is usually redissolved in water and saponins partitioned in n-butanol (Massiot et al., 1991; 1992).

Extracting saponin in butanol works well for monodesmosidic (one sugar side chain attached to aglycone) and short sugar side chain bisdesmosidic (two sugar side chains attached to aglycone) saponins. However, extraction may not be complete since bisdesmosidic saponins with several sugar units (long sugar side chain) do not readily dissolve into butanol (Oleszek, 1996), and trisdesmoside saponins like alfalfa zanhic acid cannot be extracted into butanol and are totally lost using this procedure. Oleszek (1988) reported a simple method to isolate different individual alfalfa saponins using C-18 chromatography sequentially eluted with aqueous methanol:water (1:1, 3:2, or 7:3; v:v) (Oleszek et al., 1990; 1992; Oleszek and Bialy, 2006). Eluants up to 40% methanol resulted in removal of carbohydrate and some phenolic compounds, while eluants containing 50-60% methanol removed only bisdesmoside saponins (two sugar side chains attached to the aglycone) such as medicagenic acid saponin. Eluants with 70-80% methanol eluted monodesmosides such as hederagenin and soyasapogenol saponins.

Separation of individual saponins from crude saponin extracts were achieved in a one-step procedure by Massiot et al. (1991) using thin layer chromatography on silica gel with different ratios of chloroform:methanol:water (65:35:8, 65:23:4, 60:40:3, 12:8:1, 10:10:1, 5:5:1, or 7:3:1; v:v:v). Massiot et al. (1991) also developed a procedure for separation of individual saponins based on acetylation of crude saponin mixtures followed by separation of derivatized saponins on silica gel. Chloroform:methanol, 99:1 (v:v) or combinations of chloroform:hexane:methanol (9:1:0 or 1:0:1; v:v:v) were used as solvent systems. This procedure is useful for isolation of saponins for structural work, but cannot provide pure compounds for biological assays, because the procedure cleaves some ester-linked sugars by alkaline hydrolysis prior to chromatography. Individual or pure saponins also can be isolated from saponin mixtures using HPLC on both normal- and reverse-phase columns using various solvent systems and UV detection (Kesselmeier and Strack, 1981).

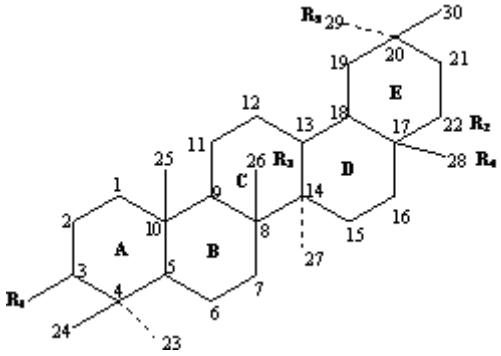
Biological Activities of Saponins

Several biological activity assays for saponins are reported in the literature (Jackson and Shaw, 1959; Van Atta et al., 1961; Coulson and Davis, 1962). The biological effects of saponins depend on their chemical structure (Figures 2 and 3). Structural variability occurs in aglycone type, carbon number, position and chemical composition (number, shape, sequence and type of monosccharides) of sugar side chains, type and number of functional groups attached to the aglycone nucleus (Potter et al., 1993).



Saponin Source	R_1	R_2	Reference
Monondesmoside yucca	+	-	Kaneda et al. (1987)
Bisdesmoside yucca	+	+	Kaneda et al. (1987)
Monondesmoside avenacin oat	+	-	Tschesche et al. (1969)
Bisdesmoside Avenacoside A, B oat	+	+	Tschesche et al. (1969)
Monondesmoside α tomatine	+	-	Haralampidis et al. (2002)

Figure 2. Steroid saponins differ in sugar side chain structure and position (R_1 and R_2) on the aglycone nucleus. + or – indicates the presence or absence of a sugar side chain, respectively.



Saponin Source	R_1	R_2	R_3	R_4	R_5	Reference
Bisdesmoside guar	+	-	-	-	+	Curl et al. (1986)
Bisdesmoside quillaja	+	-	-	+	-	Higuchi et al. (1987)
Monondesmoside soybean	-	+	-	-	-	Shirawa et al. (1991a)
Bisdesmoside soybean	+	+	-	-	-	Shirawa et al. (1991b)
Monondesmoside alfalfa	+	-	-	-	-	Massiot et al. (1988)
Bisdesmoside alfalfa	+	-	-	+	-	Oleszek (1996)
Monondesmoside alfalfa	-	-	-	+	-	Oleszek (1996)
Monondesmoside alfalfa	+	-	-	-	-	Oleszek (1996)

Figure 3. Triterpenoid saponins differ in sugar side chain structure and position (R_1 to R_5) on the aglycone nucleus. + or – indicates the presence or absence of a sugar side chain, respectively.

Guar, alfalfa, soybean and quillaja are triterpenoid saponins while yucca, oat and α tomatine are steroid saponins. The predominant guar saponins have two sugar side chains, one attached at C-3 and another at C-29 (Curl et al., 1986). The predominant quillaja saponin (Higuchi et al., 1987; 1988) and alfalfa saponins (Massiot et al., 1988; Oleszek, 1996) have two sugar side chains at C-3 and C-28. Yucca saponins (Kaneda et al., 1987; Cheeke et al., 2006), avenacoside A and B oat saponins (Tschesche et al., 1969; Grunweller and Kesselmeier, 1985; Osbourn, 2003), and soybean saponin (Kuduo et al., 1992) have two sugar side chains at C-3 and C-21. Oat saponin avenacin A-1 (Osbourn, 2003), a second yucca saponin (Kaneda et al., 1987; Cheeke et al., 2006), α tomatine in tomato (Haralampidis et al., 2002), and soyasaponin representing about 93% of the total soybean saponin (Kuduo et al., 1992) have only one sugar side chain at C-3. Hederagenin alfalfa saponin has only one sugar side chain at C-22 (Oleszek, 1996).

While saponins may share similar sugar attachment sites, the sugar side chains themselves vary in type, number, sequence, and chain length (Figures 2 and 3). Quillaja saponin contains quillaic acid as the central aglycone attached with glucuronic acid, rhamnose, hexose and a fatty acyl chain in varying ratios (Higuchi et al., 1987). Alfalfa contains medicagenic acid as the aglycone with at least one free carboxyl group, and soyasaponin I has a carboxylic group on glucuronic acid (Oleszek, 1996). Alfalfa zanhic acid saponins contain glucose attached with glucuronic acid at C-3 (Massiot et al., 1988). Avenacoside A and B (oat saponins) contain glucose and rhamnose and avenacoside B contains one more glucose unit than avenaside A (Tschesche et al., 1969).

Hemolytic Activity

Hemolytic activity has been used by researchers to follow the isolation of saponins. It is the simplest and the fastest bioassay employed to detect and quantify some saponins in plant material (Authi et al., 1988; El Izzi et al., 1992; Khalil and EI-Adawy, 1994; Onning and Asp, 1995; Oleszek, 1996; Onning et al., 1996; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001). Alfalfa (Lindahl et al., 1957; Vacek and Sedlak, 1962; Shany et al., 1970; Gestetner et al., 1971; Oleszek, 1996), soybean (Birk et al., 1963), oat (Onning et al., 1996; Pillion et al., 1996; Steurer et al., 1999), quillaja (Jenkins and Atwal, 1994), and synthetic triterpenoid or steroid saponins (Takechi et al., 1999) have hemolytic activity. Several factors determine the hemolytic activity of saponins such as aglycone type, glycone, aglycone:sugar ratio, glycone position and number of sugar side chains attached to the aglycone.

Not all saponins have hemolytic activity. For example, ginsenosides saponins hemagglutinate human, rabbit, and sheep erythrocytes, but are not hemolytic (Gogelein and Huby, 1984; McManus et al., 1993; Takechi and Tanaka, 1995a, b). Where saponins tend to have low hemolytic activity on human erythrocytes; they have high hemolytic activity on sheep cells (Schmidt-Thome and Prediger, 1950). Saponin from peas showed lower hemolytic activity than soybeans (Birk and Peri, 1980) and alfalfa root extracts are more hemolytic than those from alfalfa leaves (Shany et al., 1970).

Hemolytic activity of saponins is attributed to the affinity of their aglycone moiety to sterols within membranes, particularly cholesterol (Schmidt-Thome and Prediger, 1950; Bangham et al., 1962; Glauert et al., 1962; Joos and Ruyssen, 1967; Gee

et al., 1998; Attele et al., 1999). Other research reports that hemolytic activity decreases by removing the glycone moiety of the saponin (Santos et al., 1997). Still other researchers report that a high aglycone:sugar ratio increases hemolytic activity (Shany etal., 1970; Gestetner et al., 1971). Saponins possessing two or more sugar side chains show less hemolytic activity than saponins containing one sugar side chain (Fukuda et al., 1985; Mahato et al., 1988; Woldemichael and Wink, 2001). Takechi and Tanaka (1995b) noted that the hemolytic rates of steroid saponins are greater than the hemolytic rates of triterpenoid saponins. Santos et al. (1997) also report that steroid saponins have higher hemolytic activity than triterpenoid saponins. Monodesmoside saponins with glucose attached at C-3 shows higher hemolytic activity than monodesmoside saponins with glucuronic acid at the same position (Oleszek, 1996). As the number of monosaccharide units attached to the hydroxyl group at C-3 on the aglycone (Mahato et al., 1988) increases up to four to six sugar units (Anisimov et al., 1980; Kuznetzova et al., 1982) hemolytic activity increases. The stereochemistry of the saponin as related to side chain composition and length appear to be very important in conferring activity on the saponin molecule (Gee et al., 1998).

Hemolytic activity of saponins increases with decreasing numbers of polar groups on the aglycone moiety (Namba et al., 1973). Saponins with the highest retention factor (R_f) on silica gel showed the strongest hemolytic activity. The active groups on the aglycone and the acylation of saponins affect hemolytic activity. The neutral and acidic triterpenoids along with the acyl saponins are less hemolytic than the ester saponins (Gee et al., 1998; Attele et al., 1999; Oda et al., 2000). Laurence et al. (2005)

noted that the ethanol acylated triterpenoid saponins isolated from the stem bark of *Harpullia austro-caledonica* showed 100% hemolytic activity of a 10% suspension of sheep erythrocytes while, deacylated quillaja saponins, which differ only in the absence of one glucose residue decreased hemolytic activity (Pillion et al., 1996). No close and direct relationships between hemolytic activity and other biological activities of saponin were reported. For example, avenacoside oat saponins showed hemolytic activity, but did not affect the cell membrane permeability and the active transport of glucose (Abe et al., 1978a; Onning et al., 1996; Pillion et al., 1996; Steurer et al., 1999). This is a very unusual observation given hemolysis is the result of a ruptured red blood cell membrane which allows hemoglobin to escape the cell. A trisdesmoside zahnic acid saponin was only weakly hemolytic and neither inhibited fungal growth nor formed insoluble complexes with cholesterol (Gee et al., 1996). However, it was the most active compound affecting cell membrane permeability and active transport of glucose, giving further evidence of the complexity of the interactions between saponins and membranes (Gee et al., 1996). Synthetic steroid saponins show both antifungal and hemolytic activity, but in many cases hemolytic triterpenoid saponins show little antifungal activity (Takechi et al., 1999).

Antibacterial and Antiprotozoal Activities

As with hemolytic activity, the antibacterial activity of saponins is affected by factors such as the aglycone, number, position and chemical structure of sugar side chains (Rakhimov et al., 1996). Many saponins are antimicrobial and considered as a

part of plants' defense systems. Plants known to have antimicrobial activity include yucca (Hussain and Cheeke, 1995; Tanaka et al., 1996), quillaja (Sen et al., 1998b), ginseng (Mahato et al., 1988), and triterpenoid saponins from *Holothuroidea* class of marine echinoderm animals (Samoilov and Girshovich, 1980). Not all saponins have antibacterial activity. For example, medicagenic and zanhic acid saponins isolated from alfalfa plants do not show activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtitles*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare* (Oleszek and Jurzysta, 1992). Yucca saponin show moderate antibacterial activity against gram-positive bacteria such as *Staphylococcus aureus* and *Lactobacillus plantarum* but are without antibacterial activity against gram-negative bacteria such as *Escherichia coli* (Tanaka et al., 1996).

Antibacterial activities of saponins differ according to type of the bacteria. Some saponins such as ivy saponin (Cioaca et al., 1978), spirostanol saponin (Okunji et al., 1990), asterosaponin from starfish (Andersson et al., 1987; 1989), and yucca saponin (Cheeke, 1998; Cheeke and Otero, 2005) show more antimicrobial activity against gram-positive bacteria (*Staphylococcus aureus*) than gram-negative bacteria (*Escherichia coli*) at the same concentration. Wang et al. (2000a, b) note that saponin-rich yucca extracts in ruminant diets decreased cellulolytic bacteria while not affecting amylolytic bacteria.

Quillaja saponin (*Quillaja saponaria*) and yucca saponin (*Yucca schidigera*) obtained from different commercial companies exhibited antibacterial activity against *Escherichia coli* K-12 with different efficiencies, suggesting that saponins from various sources differ in their biological activity due to their different chemical structures and

extraction procedure (Sen et al., 1998b). Extraction methods and fat content have important effects on the antibacterial activity of the resultant plant extracts. For example, fat free extracts from *Bauhinia variegata* L. bark were more active than high fat extracts against gram-positive bacterial strains such as *Staphylococcus aureus* (Rakhimov et al., 1996). However, fat free extracts exhibited either similar or less antibacterial activity than high fat extracts against gram-negative bacterial strains such as *Escherichia coli*.

Many saponins have antiprotozoal activity (Lu and Jorgensen, 1987; Wallace et al., 1994; Newbold et al., 1997; Cheeke et al., 2006) as a result of binding with sterols present on the protozoal surface, which are absent on bacterial membranes (Hussain and Cheeke, 1995). The sugar side chain attached to the hydroxyl group on C-3 has an important effect on the antiprotozoal activity of many saponins. The antiprotozoal property of saponins is lost upon deglycosylation (Wang et al., 2000a).

Effect of Saponin on Cell Membrane Permeability

Saponins differ in their effects on cell membrane permeability. Many reports suggest negative effects on cell membrane permeability by blocking membrane ion channels (Segal et al., 1974; Abe et al., 1978b; Matsuda et al., 1997; Santos et al., 1997) and irritating membranes of the mouth and the digestive tract (Oleszek and Jurzysta, 1992; Oleszek et al., 1994). Other research reports that saponins increase cell membrane permeability (Price et al., 1987; Gee et al., 1989; Brain et al., 1990) by insertion of the aglycone into the lipid bilayer (Hu et al., 1996), forming pores of 40 to 50A° in diameter in human erythrocyte membranes (Seeman et al., 1973; Seeman, 1974; Authi et al.,

1988; El Izzi et al., 1992; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001). Saponins also have stimulated sodium–calcium exchange activity as in canine cardiac sarco-lemmal vesicles (Yamasaki et al., 1987; Choi et al., 2001) and induce an isotropic action through membrane calcium channels (Enomoto et al., 1986). Saponins change the function of proteins or glycoproteins in plasma membranes (Abe et al., 1978b; Rao and Sung, 1995), and form a saponin–cholesterol complexes therby altering the organization of membrane phospholipids, form phospholipid breakdown products such as phosphatidic acid (Yamasaki et al., 1987; Choi et al., 2001). ATPases enzyme activity of membranes also is altered thereby affecting ion transport (Ma and Xiao, 1998).

In general, saponin action on cell permeability is dependent on concentration and the number of sugar side chains on the aglycone. High concentrations of saponin may literally poke holes in cell membranes while lower concentrations may interact with membranes in other ways without actually rupturing them. The permeability activity of avenacin A-1 is completely abolished after one, two, or all three sugar side chains are hydrolyzed to yield monodeglucosyl, bisdeglucosyl and aglycone derivatives, respectively (Armah et al., 1999).

Other Biological Activities of Saponins

In addition to the previously mentioned biological activities of saponins, they are also reported to have anti-arthritic (Cheeke et al., 2006), anthelmintic (Julien et al., 1985; Tanaka et al., 1996), diuretic (Sood et al., 1985), molluscicidal (Lemma, 1965), insecticidal (Applebaum et al., 1965; 1969), hypolipidaemic and hypocholesterolemic

(Malinow et al., 1977; Malinow, 1984; Al-Habori and Raman, 1998). Other studies reported several anticancerous biological activities of saponins (Ma et al., 2007). Saponins also can induce diarrhea (Lalitha et al., 1990), mollify ulcers (Muto et al., 1987), relieve analgesia (Lei et al., 1984; Oshima et al., 1984) and fever (Gan and Chen, 1982), as well as having sedative properties (Wagner et al., 1983; Choi and Woo, 1987). Other report antidiabetic (Kamel et al., 1991; Al-Habori and Raman, 1998), antifungal (Shimoyamada et al., 1990; Takechi et al., 1999; Lee et al., 2000; Yang et al., 2006), antiyeast (Kasai and Yamasaki, 2000; Tanaka et al., 2000) and antimold (Morrissey and Osbourn, 1999) activities. Saponins exhibited anti-inflammatory (Chevillard et al., 1965; Haridas et al., 2001a, b; Cheeke et al., 2006; Jigna et al., 2006), spermicidal and contraceptive (Elbary and Nour, 1979; Bhargava, 1998a, b), antioxidant (Monder et al., 1989; Pawar et al., 2001; Hu et al., 2002) and antiviral (Sindambiwe et al., 1998; Apers et al., 2001) activities.

Many of the biological activities mentioned above can be mediated by interaction of saponins with cell surface proteins and receptors, secondarily affecting enzymes. Many saponins affect inhibit enzyme activities such as urease (Lyons, 1989), sodium-potassium ATPase, calcium–magnesium ATPase in rabbit red blood cell membranes (Kang and Koh, 1974; Choi et al., 2001) and mucosal enzyme activity in the small intestine (Olli et al., 1994).

Saponins also affect hormone activity. For example, saponins exhibit strong stimulation effects on both adrenocorticotropic hormone (ACTH) and corticosterone hormone (Kim et al., 1999). They show mineralocorticoid-like activity by transforming

biologically active steroid cortisol hormone into its inactive metabolite, cortisone (Monder et al., 1989). Glucocorticoid hormones that act through glucocorticoid receptors (Gagliardo et al., 2001) and plasma insulin hormone concentrations (Petit et al., 1993), while, other saponins showed inhibitory effects on insulin hormone (Akhtar and Perry, 1975).

They also stimulate uterine growth, block the estrous cycle (Benie et al., 1990), improve the copulatory performance of sexually sluggish or impotent rats by increasing central noradrenergic, dopaminergic tone and oxytocinergic transmission (Arletti et al., 1999), compete strongly with estradiol for estrogen- and progesterone-binding sites in the human myometrial cytosol on uterine receptors (Punnonen and Lukola, 1980; Benie et al., 1990). Other effects include stimulating binding of progesterone to bovine luteal membrane by forming complexes with membrane sterols (Menzies et al., 1999), inhibiting the activity androgen receptors by binding to androgen receptors (Liu et al., 2000), prolonging the period of diestrous (Tamura et al., 1997), inhibiting the genes responsible for steroidogenesis and suppressing the proliferation of follicle-stimulating hormone-modulated granulosa cells in the ovarian follicle through a similar mechanism as saponin-induced proliferation of tumor cells (Tamura et al., 1997).

Toxicity of Saponin

Many saponins exhibit toxic effects at high doses over long periods of time causing problems such as excessive salivation, vomiting, diarrhea, loss of appetite and manifestations of paralysis (Spinks and Fenwick, 1990). Oral toxicity of saponins to

warm-blooded animals is relatively low (George, 1965) and LD_{50} (lethal dose, 50%) values are in the range 50-1000 mg/kg (Oakenfull, 1981). However, they are highly toxic when given intravenously (Makkar and Becker, 1996). The toxic effects of many saponins are neutralized by saliva of animals such as sheep (Odenyo et al., 1997; Teferedegne, 2000), intestinal bacteria (Price et al., 1987; Newbold et al., 1997; Odenyo et al., 1997; Wakabayashi et al., 1998; Bae et al., 2000; Park et al., 2001), and rumen bacteria (Newbold et al., 1997; Flaoyen et al., 2001). Cooking or heat processing can also detoxify saponins. For example, cooking decreases saponin content by 7-17% in chick peas, 40% in faba bean seeds (Sharma and Sehgal, 1992) and 72% in quinoa (Gee et al., 1993). Not all saponins are degraded at the same conditions and temperature. For example, oat saponins are not affected (Kataria et al., 1989) until heated to 140^{o}C for 3 h. Degradation increases as the pH decreases from 7 to 4 in avenacoside A (Onning and Asp, 1995). Saponin degradation sometimes induces activity of enzymes such as β-glycosidase that occur naturally in oat leaves. Removing the C-26-bound glucose moiety (Grunweller and Kesselmeier, 1985) results in forming a monodesmosidic saponin with the highest antifungal activity. Also, avenacosides saponin A and B in oats are activated by the plant's enzymes in response to tissue damage or pathogen attack by fungi (Gus-Mayer et al., 1994; Hostettmann and Marston, 1995; Sauvaire et al., 1996). Saponins can also be hydrolyzed by cleaving the ester-linked sugars to yield carbohydrates and aglycones (Oleszek et al., 1990; 1992; Levy et al., 1989; Oleszek and Bialy, 2006).

Effect of Saponins on Intestinal Activity, Nnutrient Digestion and Aabsorption

Saponins reduce intestinal motility (Klita et al., 1996), increase the transit time of ingesta (Ueda et al., 1996), inhibit gastric emptying (Matsuda et al., 1999a, b; Yoshikawa et al., 2001). Saponins can cause intestinal lesions (Bangham et al., 1962; Seeman, 1974), damage the intestinal villi (Story et al., 1984; Gee and Johnson, 1988; Gee et al., 1993; Bureau et al., 1998) and alter intestinal morphology in the lower intestine (Baker et al., 1988).

Many saponins decrease digestion by changing the site and the extent of nutrient digestion in ruminants and altering rumen fermentation (Lu and Jorgensen, 1987; Lu et al., 1987; Killeen et al., 1998). Total volatile fatty acid and microbial protein synthesis can be reduced (Lu et al., 1987). Digestive coefficients of some organic matterials such as hemicelluloses and cellulose are reduced and digestibility of some nutrients such as protein are also reduced perhaps potentianted by damage to the intestinal membranes (Freeland et al., 1985; Potter et al., 1993; Shimoyamada et al., 1998).

Although, saponins remain within the gastrointestinal tract and are not absorbed into the blood stream (Birk, 1969; Yoshikoshi et al., 1995; Cheeke, 1996), perhaps because of their binding to the intestinal mucosa (Gee and Johnson, 1988), many saponins inhibit the absorption of several nutrients (Johnson et al., 1986) such as vitamins A and E (Jenkins and Atwal, 1994). On the other hand, many saponins have been reported to increase the absorption of certain substances (Freeland et al., 1985) such as calcium, iron salts, glucose, acetylsalicylic acid, magnesium, sodium, sodium

ampicillin and other β-lactams in rat and pig intestine (West, 1979; Kimata et al., 1983; Lower, 1984; Freeland et al., 1985; Yata et al., 1985; Southon et al., 1988b).

Effect of Dietary Saponins on Poutry and Animal Performance

Some saponins showed beneficial effects on the productive performance of poultry and animals. For example, yucca and quillaja saponins are classified as generally recognized as safe (GRAS) for food use in the USA (Fenwick et al., 1992). Yucca saponins are active over a wide pH range (3.0-6.3), without toxic effects, safe for human food, tasteless, odorless, soluble in water, and stable upon heating (Tanaka et al., 1996). Adding yucca saponin to diets might be beneficial to both ruminants and chickens fed high-grain diets by reducing the emission of ammonia from animal excreta (Lyons, 1989; Wang et al., 2000a, b; Cheeke and Otero, 2005). Also, quillaja saponins increase the efficiency of rumen-microbial protein synthesis in vitro (Makkar and Becker, 1996). Saponins reduce the toxicity of some toxins such as tannins by binding them in the gut (Freeland et al., 1985).

The beneficial effects of some saponins on productive performance include improving egg production in laying hens, enhancing viability, increasing growth rate and feed efficiency in broilers and pigs (Johnston et al., 1980; 1981; 1982; Mader and Brumm, 1987; Al-Bar et al., 1993; Anthony et al., 1994; Killeen et al., 1998), increasing feed and water intake (Podgorski and Majewski, 2002), reducing O_2 consumption thus improving metabolic rate and nutrient utilization (Francis et al., 2001a, b; 2002a, b). Other researchers report reducing NH_3 concentration in the rumen and plasma (Hussain

and Cheeke, 1995; Cheeke and Otero, 2005), stimulating the appetite (Petit et al., 1993; Sauvaire et al., 1996) and inducing fattening of lambs and steers (Hale et al., 1961; Goodall et al., 1982). Saponin effects in lambs are sex-dependent. Bosler et al. (1997) found that both male and female lambs fed up to 40 mg quillaja saponin/kg increased average daily weight gains, but that the difference in weight gain was lower in the females than males. Beneficial effects of saponins were more pronounced when they were directly administered into the rumen rather than added to the feed (Odenyo et al., 1997).

Many saponins have harmful effects on productive performance of monogastric and ruminant animals. Alfalfa saponins are of limited use, especially in monogastric animals such as swine, fish and poultry for their anti-nutritional effects (Newman et al., 1958; Sim et al., 1984; Terapunduwat and Tasaki, 1986; Price et al., 1987; Mahato et al., 1988; Shimoyamada et al., 1990; Potter et al., 1993; Jenkins and Atwal, 1994; Olli et al., 1994; Makkar and Becker, 1996; Forhne, 1999; Francis et al., 2001a, b) partially because of decreased palatability. Decreased feed intake, growth rate (Cheeke, 1983; 1996; Ueda and Ohshima, 1987; Makkar and Becker, 1996), increased mortality rate (Lalitha et al., 1990), and depressed egg production in poultry and fish result (Sim et al., 1984; Terapunduwat and Tasaki, 1986; Potter et al., 1993; Jenkins and Atwal, 1994; Francis et al., 2001a, b). They also are reported to irritate mucous membranes of the mouth, digestive and respiratory tracts (Forhne, 1999).

Some saponins also exert harm to animal reproductive performance (Tewary et al., 1973; Stolzenberg and Parkhurst, 1976) by causing abortion or death in rabbits, goats

and cows (Dollahite et al., 1962), preventing pregnancy (Tewary et al., 1973; Mahato et al., 1982; Quin and Xu, 1998) and inducing proliferative changes in both the vagina and uterus (Chou et al., 1971; Tewary et al., 1973; Stolzenberg and Parkhurst, 1976).

On the other hand, some saponins are harmful, but many of them are beneficial (Wallace et al., 1994; Hussain and Cheeke, 1995). For example, alfalfa and soybean saponins have both beneficial and adverse effects in many animals (Sen et al., 1998a, b). Saponins even affect animal species differently. Various avian species show an aversion to alfalfa meal (Cheeke, 1983) and sheep appear less sensitive than cattle to saponins' bitter taste (Cheeke, 1994). Specific tissues also are affected by saponins. The weights of some internal organs such as the adrenal glands are increased while other saponins decrease the weight of the thymus in rats (Hiai and Yokoyama, 1986; Hiai et al., 1987). Some ginseng saponins increase motility and progression of sperm (Chen et al., 2001), while *Sesbania sesban* saponins are spermicidal in vitro (Dorsaz et al., 1988).

Effect of Saponin on Feeding Behavior

Saponins can affect the circadian rhythm of feeding behavior (Petit et al., 1995). Rats fed steroid saponin-rich fenugreek seed extract ate continuously during 24 h rather than only at night. Normally, rats consume 80-90% of their nutritional intake at night, a consumption pattern which was stable throughout the saponin treatment. In contrast, food intake increased two-fold during the daylight phase with the steroid saponin treatment (Petit et al., 1995).

Other studies reported no adverse effects of adding saponin in the diet of monogastric animals (Ishaaya et al., 1969; Mahato and Nandy, 1991; Wu et al., 1994). Steroid saponins from yucca or triterpenoid saponins from soybean did not affect growth rate at concentrations as much as five times the concentration of saponin from soybean-supplemented diets of chicks, rats and mice. Quillaja saponin did not show significant toxic effects in short-term feeding studies in rats (Gaunt et al., 1974) or long term toxicity studies in mice (Philips et al., 1979).

Guar Meal Is a Saponin-Rich Legume

Guar, *Cyamopsis tetragonoloba* L. (syn. *C. psoraloides*) or cluster bean is a drought-tolerant summer annual legume native to India and Pakistan where it is cultivated as a feed crop for human and livestock consumption (Rahman and Shafivr, 1967). Guar was initially introduced by the USDA in 1903 as a feed crop appropriate for arid areas of the USA. Currently guar is produced in India, Pakistan, and the USA with smaller quantities of production in Australalia and South Africa (Udersander et al., 1997). Its production is centralized in northern Texas and southwestern Oklahoma, because of suitable climate and soil type (Rahman and Shafivr, 1967; Whistler and Hymowitz, 1979).

Guar plant's growth characteristics make it beneficial for arid production areas. Guar is drought resistant, does not require irrigation, and adapts to many soil types. It is a legume commensal with nitrogen fixing bacteria within guar root nodules. Therefore, guar is a good rotation plant with cotton and other crops. When plowed under, guar

increases crop yield due to the improved nitrogen availability in soil (Whistler and Hymowitz, 1979; Undersander et al., 1997).

Refinement and Separation Processes of Guar Beans

The guar bean is divided into three fractions, endosperm, hull and germ that compose 35-42, 14-17 and 43-47% of the whole bean, respectively. Mechanical refinement of the guar bean is initiated by pouring whole raw guar beans into a set of attrition mills to crack the beans. Broken beans are conveyed through a set of 14 mesh sieves. At this point, the guar germ fraction is removed as it is the only particle size small enough to pass through the screen. This portion is now termed ″14 mesh guar meal.″ The remaining fraction now termed crude cracks, are then passed through a rotary furnace between 93.3 and 105^{o}C. Heat helps to separate guar endosperm and hull fractions. Crude cracks are passed through a second set of mills known as micro mills where the endosperm remains a large particle and the hull is shattered. These products are passed over 18 mesh sieves. At this point, the guar endosperm and the hull fractions are separated. The guar hull fraction is termed ″18 mesh guar meal″ and the guar endosperm fraction is termed ″splits″. Endosperm fractions, splits, are stored for further processing of guar gum. Guar gum production yields guar germ and hull fractions as by-products. The 18 mesh guar meal (guar hull fraction) is combined with 14 mesh guar meal (guar germ fraction) to form guar meal.

Dietary Guar Gum and Guar Meal Effects on Animal and Poultry Performance

Guar Gum

Currently, guar bean is mainly produced for the guar gum, a galactomannan polysaccharide that is contained in the endosperm. Guar gum is about 13-18% of the original weight of the guar bean (Bakshi et al., 1964, Lee et al., 2004). Chemically, guar gum is a linear chain of D-mannose units connected by β-1-4 glycoside bonds. Every other D-mannose unit bonds a D-galactose unit by α-1-6 glycoside linkage. Commercial guar gum is composed of approximately 8-14% moisture, 75-85% galactomannan, 5-6% crude protein, 2-3% crude fiber and 0.5-1.0% ash (Maier et al., 1993). It is utilized as a thickening agent in paint, cosmetics, and pharmaceuticals. Guar gum also is utilized in oil well drilling mud, cloth and paper, explosives, ore flotation, as well as a variety of other industrial uses (Undersander et al., 1997). Guar gum can be used to control water loss, viscosity, flocculation, suspension, and mobility (Whisler and Hymowitz, 1979).

Guar gum has a variety of uses in food products as a binder or thickening agent. A typical analysis of food grade guar gum includes 2.5% crude fiber, 1-5% crude protein, and 0.5-0.8% ash (Whisler and Hymowitz, 1979). Guar gum can be found in food products such as candy, ice cream, canned meats, pasta, instant puddings, colas, and as a stabilizer for cheeses. Guar gum improves texture and enhances food flavor by controlling moisture distribution and migration (Whisler and Hymowitz, 1979). Guar gum is useful in frozen foods, such as ice cream as a stabilizer and water-binder. It is

also useful as a stabilizer in baking and in high-temperature, short-term processing of bread (Whisler and Hymowitz, 1979).

High concentration of guar gum in poultry diets lowers absorption of amino acids through the intestinal wall (Katoch et al., 1971). The growth depressing properties of guar gum can be mitigated by treatment with enzymes capable of hydrolyzing guar gum. Pectinase, cellulase or a preparation from sprouted guar beans have been used to digest guar gum in poultry (Vohra and Kratzer, 1964; 1965) and pig diets (Chesson, 1987).

Guar gum can dramatically alter the ecological properties of the contents of the gastro-intestinal tract resulting in significant physiological effects. High viscosity is generally observed with delayed gastric emptying and increased small intestinal transit time, hence inhibiting the nutrient absorption (Blackburn and Johnson, 1981). However, high viscosity guar gum may contribute to beneficial physiological functions in human health by decreasing plasma cholesterol (Favier et al., 1998; 1997; Dario Frias and Sgarbieri, 1998; Moriceau et al., 2000; Yamamoto et al., 2000) and decreasing serum glucose (Groop et al., 1993; Fairchild et al., 1996; Ou et al., 2001; Russo et al., 2003).

Guar gum also has putative protective effects against colonization by pathogenic bacteria. Pathogenic bacterial proliferation and the resultant diarrhea can be ameliorated by saponins. The polar lipid effect prevents microbial translocation within the gastro-intestinal tract (Bengmark, 1998). Guar gum is readily fermented by human fecal microbes (Okubo et al., 1994), which may partially contribute to a prebiotic function. Duncan et al. (2002) successfully isolated a high-molecular-weight galactomannan similar to guar gum from the edible mushroom *Morchella esculenta* that enhances

macrophage activation thus exhibiting immunostimulatory activity. Guar gum could also have such effects.

A series of feeding experiments conducted by Vohra and Kratzer (1964) demonstrated that as little as 1% guar gum in broiler chicken diets causes a depression of growth. When the diet contained 2% guar gum, relative growth of broiler chickens was 61-67% of controls. Residual gum was throught to be chiefly responsible for the adverse effects on chicks, not trypsin inhibitor (Anderson and Warnick, 1964; Vohra and Kratzer, 1964) which was shown to be insignificant (Conner, 2002). Lee et al. (2003a) also supported that residual gum was at least partially responsible for the negative growth effects seen when guar meal was fed.

Guar Meal

Estimates of total world production of guar meal in 1975 were between 400 and 500 million pounds as animal feed (Whisler and Hymowitz, 1979). India, the major producer of guar that accounts for 80% of the total guar produced in the world produced betwwen 200 to 600 thousand tons of seed and between 400 to 500 thousand tons of guar splits in 2007. The world market for guar gum is estimated to be around 150,000 tons/year, 70% of which is produced by India and Pakistan (Rediff India Abroad, Sep, 2007). Guar meal contains about 33-47.5% crude protein on a dry matter basis (Bakshi, 1966; Ambegaokar et al., 1969). This protein source is valuable as poultry and animal feed. However, guar meal's use in poultry and pig rations historically was limited by its adverse effects on feed intake and growth (Thakur and Pradhan, 1975a, b). Guar meal

primarily has been used in ruminant feeds, particularly for beef cattle in feedlots in northern Texas and southwest Oklahoma. Guar meal has not been used extensively with monogastric animals because of perceived growth retarding characteristics of the meal. Guar meal at high concentrations has deleterious effects on growth rate and feed efficiency of broiler chicks (Brocher and Ackerson, 1950; Saxena and Pradhan, 1974). Guar meal is sticky in nature and reduces nitrogen retention, energy utilization, fat absorption from the gut, depresses growth rate and increases mortality rate in broilers (Anderson and Warnick, 1964). Despite these deleterious effects, the low cost of guar meal make it a potentially good source of essential amino acids (Ramakrishnan, 1957).

The current market value of guar meal is approximately 7.5-10 cents per pound (West Guar-Tex and Rhodia Companies). Other feed ingredients such as cottonseed meal, canola meal and soybean meal containing comparable crude protein values have current market values between 16.0-18.8 cents per pound (USDA Market News, Feb., 2008).

Verma and McNab (1984a, b) reported that protein in guar meal is rich in arginine, but methionine and lysine concentrations were inadequate for optimum rat growth and comparatively lower than concentrations typically found in soybean meal (Van Etten et al., 1961). Ambegaokar et al. (1969) suggested that tryptophan, methionine and threonine were the first three deficient amino acids of guar meal when compared to whole egg protein. Excessive concentrations of guar meal in poultry diets cause diarrhea, depresses growth rate and increases mortality of broilers (Sathe and Bose, 1962; Couch et al., 1967a; Thakur and Pradhan, 1975b; Verma and McNab, 1982; Patel and

McGinnis, 1985; Conner, 2002) and decreases feed efficiency of laying hens (Bakshi et al., 1964; Nagra et al., 1985; Patel and McGinnis, 1985; Nagra and Virk, 1986).

Guar meal can be safely fed to laying hens at concentrations up to 5% without adverse effects on feed efficiency (Cheng, 2004). He reported also that feeding 10% guar meal depressed feed consumption and increased body weight loss. Reduced feed intake produces a molting effect when used at 20% of the diet and can thus be considered an alternative molting strategy that does not require complete removal of feed. Bakshi et al. (1964) proposed that guar meal contains two deleterious factors: trypsin inhibitor and guar gum residue. The trypsin inhibitor was listed as a deleterious factor because the chicks fed guar meal had been reported to present pancreatic hypertrophy (Couch et al., 1966; 1967b) which can also be found in chickens fed un-heated soybean meal. However, the trypsin inhibitor is not universally accepted as a primary factor for the deleterious effects of feeding guar product to poultry (Anderson and Warnick, 1964; Vohra and Kratzer, 1964). Verma and McNab (1982) reported that neither heating the guar meal directly nor steam pelleting diets containing guar meal had much effect on the performance of the broiler chicks, which was in agreement with the findings of Nagpal et al. (1971) who reported autoclaving guar meal did not improve its gross protein value for chicks. The trypsin inhibitor activity in guar meal was reported to be significantly lower than in soybean meal commonly used in poultry feed (Verma and McNab, 1982; Conner, 2002), which indicates that the harmful effects on performance of poultry when fed diets containing guar meal are not likely due to excessive trypsin inhibitor activity.

Guar meal contains 5-13% triterpenoid saponin of dry matter (Curl et al., 1986; Hassan et al., 2007). The predominant guar saponins have two sugar side chains, one attached at C-3 and another at C-29 (Curl et al., 1986). Guar meal also contains guar gum whose impact on intestinal viscosity affects growth and feed efficiency (Lee et al., 2003a, b; 2004; 2005).

Research Description

The previous information reviewed indices that new antibiotic and coccidiostats must be continually developed to overcome increasing resistance to chemical drugs in bacteria and parasites (Chapman, 1993; 1998; Yadav and Gupta, 2001). Drug resistance and the high costs of developing novel drugs make discovery of alternative control methods essential (Chapman, 1997). Antibiotics have been used extensively in animal feed to stimulate animal growth rate and to inhibit growth of intestinal pathogens. However, continued feeding of antibiotics at sub-therapeutic levels has created concerns about the extent to which usage increases antibiotic residue in food, develops drug resistant bacteria, and reduces the ability to cure bacterial infections in humans (Jensen, 1998). Antibiotic use in animal production is no longer desirable because of concerns about increasing antibiotic resistant pathogenic microorganisms in both veterinary and human medicine (Nawaz et al., 2000; Bertolatti et al., 2003; Wallace, 2004).

Increased awareness of potential problems associated with the use of antibiotics has stimulated research efforts to identify alternatives to antibiotics used as feed additives. Novel approaches to the development of antimicrobial compounds and

materials are probiotics, prebiotics (Murry et al., 2004), organic acids (Van Immerseel et al., 2006), medical herbs (Wink, 1999; Gordon and David, 2001; Du and Hu, 2004; Arab et al., 2006) and plant extracts. Many potential antibacterial compounds (Sen et al., 1998b; Avato et al., 2006) have not been surveyed adequately for effectiveness.

The intent of this study is to determine the anticoccidial, anticlostridial and growth effects of guar meal and its saponin-rich extracts on biological activities evaluated by using hemolytic and antibacterial assays. To date, no research has evaluated the biological activities of saponin-rich guar meal extract. In this study, the biological activity of the guar meal and its saponin-rich extracts focused on four pathogenic gram-negative and gram-positive bacteria (*Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli*, and *Clostridium perfringens*), one pathogenic protozoan (*Eimeria tenella*) and one beneficial gram-positive bacterium (*Lactobacillus spp.*). Four experimental studies were conducted. Therefore, the first study was conducted to isolate several fractions of saponin-rich guar meal extract and evaluate these extracts for hemolytic and antibacterial activities against *Staphylococcus aureus, Lactobucillus* spp., *Escherichia coli* and *Salmonella* Typhimurium in a dose dependent manner. The second study was conducted to evaluate the hemolytic and antibacterial activities of saponin-rich guar meal against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium in comparison to three common commercial saponin sources (yucca, quillaja and soybean) in a dose dependent manner. The third study was conducted to determine the anticoccidial effect of adding 5% guar meal in a corn-soy based broiler starter diet in chicks challenged by *Eimeria tenella*.

Body weight, feed conversion ratio, mortality rate, duodenal pH, cecal lesion score, and number of oocysts per gram of feces were measured. The fourth study was conducted to determine whether addition of either 2.5% guar meal, 1% guar gum or 0.125% saponin-rich guar meal extract in 55% wheat-corn-based broiler starter diets would ameliorate lesions of necrotic enteritis in broiler chickens challenged with *Clostridium perfringens*.

CHAPTER III

ANTIMICROBIAL AND HEMOLYTIC ACTIVITIES OF SAPONIN-RICH EXTRACTS FROM GUAR MEAL

Introduction

Antibiotics used in animal production are no longer desirable because of concerns about bacterial resistance (Wallace, 2004). Increased awareness of the potential problems associated with the use of antibiotics stimulates research efforts to identify alternatives to their use as feed additives. Novel approaches to the development of new antimicrobial like compounds to treat diseases or to improve poultry growth include dietary use of probiotics, prebiotics (Drake et al., 2003; Patterson and Burkholder, 2003; Murry et al., 2004; Higgins et al., 2005b; 2007; Tellez et al., 2006), organic acids (Van Immerseel et al., 2006), medicinal herbs (Wink, 1999; Gordon and David, 2001; Du and Hu, 2004; Arab et al., 2006), non-traditional chemicals (Moore et al., 2006) and bacteriophages that are bacteria-killing viruses (Higgins et al., 2005a; Vicente et al., 2005).

Original candidate chemical structures for many pharmaceutical compounds that promote human health originated from chemicals found in plant extracts (Du and Hu, 2004; Arab et al., 2006). Among these plant compounds are saponins from quillaja and yucca which have beneficial effects on animal health (Sen et al., 1998b; Avato et al., 2006). Saponins are glycoside compounds whose chemical structures are composed of a fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) or neutral or alkaloid

steroid (C-27) attached to one or more side chains of water-soluble sugars (glycone) through ester linkages to the aglycone nucleus at different carbon sites. Triterpenoid saponins predominate in soybean, alfalfa, quillaja and guar while steroid saponins predominant in yucca, tomato and oats (Tschesche et al., 1969; Kaneda et al., 1987; Haralampidis et al., 2002).

Among the various biological effects of saponins are hemolytic activity (Schmidt-Thome and Prediger, 1950; Joos and Ruyssen, 1967; Seeman et al., 1973; Fukuda et al., 1985; Khalil and El-Adawy, 1994; Woldemichael and Wink, 2001) and antibacterial activity (Mahato et al., 1982; Sen et al., 1998a, b). Some saponins are suspected to be beneficial while others are considered hazardous to animals. Biological activities of saponins are affected by factors such as the saponin nucleus type, number of sugar side chains and type of functional groups (Cheeke, 1998; Osbourn, 2003).

Guar, *Cyamopsis tetragonoloba* L. (syn. *C. psoraloides*) or cluster bean is a drought tolerant annual legume grown primary for the guar gum (galactomannan polysaccharide) that has many industrial and food processing applications. Guar meal is a combination of hull and germ fractions that is produced as a by-product of guar gum manufacture. Guar is used in some parts of the world, primarily India and Pakistan, as a human and animal food. Guar also contains 5-13% crude saponin by weight of dry matter (Curl et al., 1986; Hassan et al., 2007) and thus contains potentially useful bioactive compounds. No research has evaluated the biological activities of guar saponin. Therefore, this study reports isolation of saponin-rich guar meal extract and evaluates these extracts for hemolytic and antibacterial activities against two gram-

positive bacteria (*Lactobacillus spp.* and *Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli* and *Salmonella* Typhimurium) in a dose dependent manner.

Materials and Methods

Isolation of Saponin-Rich Guar Extract

Guar meal (Rhodia Inc., Vernon, TX) was ground in a commericial coffee mill to form a fine powder and subsequently extracted by refluxing 25 g of guar meal with 250 mL of ethanol:water, 1:1 (v:v) for 3 h in a simple reflux apparatus according to the method of Livingston et al. (1977) and Khalil and El-Adawy (1994). Refluxed extracts were cooled and filtered through 150-µm (Watman No. 2) then 125-µm pore size (Watman No. 114) filter papers. Ethanol was removed from the filtered extract by evaporating under reduced pressure in a roto-evaporator (Buchi, Rinco Instrument Co., Inc., Greenville, IL, Switzerland, model 310391) until two-thirds of the initial volume was removed. The remaining aqueous extract was partitioned with n-butanol, 1:1 (v: v) overnight at room temperature using a separatory funnel. The upper n-butanol extract was collected in a glass flask and the lower aqueous extract was collected and further partitioned with n-butanol two more times to increase the yield of crude saponin. The butanol extracts were pooled and evaporated to dryness using the roto-evaporator procedure described above. A minimum volume of water was added to the dry n-butanol

extract and the resulting material was freeze-dried, weighed and stored at room temperature until used in further studies.

Preparative Chromatography of Guar Saponin

A 40 mm x 150 mm inner diameter flash chromatography column was packed using 30 g of 230-400 mesh C-18 media (EMD Chemicals Inc.) in 100% iso-propanol. The column was incorporated into a Bio Rad Biologic chromatography workstation with a model 2128 fraction collactor and equilibrated with isopropanol for 5 min at a flow rate of 30 mL/min. Freeze-dried n-butanol extract was dissolved into distilled water and injected onto the preparative reverse-phase column and developed with a step gradient of methanol:water (20:80, 60:40 and 100:0, (v:v), respectively over a 96 min run time at a flow rate of 4 mL/min. The 20% methanol mobile-phase eluted a yellow and orange peak, presumably rich in flavanoids. The 60% and 100% mobile-phase eluted two colorless peaks (Figure 4). Peaks eluting with 20, 60, and 100% MeOH were pooled separately into 3 fractions designated 20% MeOH, 60% MeOH and 100% MeOH and evaporated to dryness using the roto-evaporator. A minimum volume of water was added to each dry methanol extract and the resulting material was freeze-dried, separately weighed and stored at room temperature until needed. The procedure was repeated to obtain sufficient pooled semi-purified material.

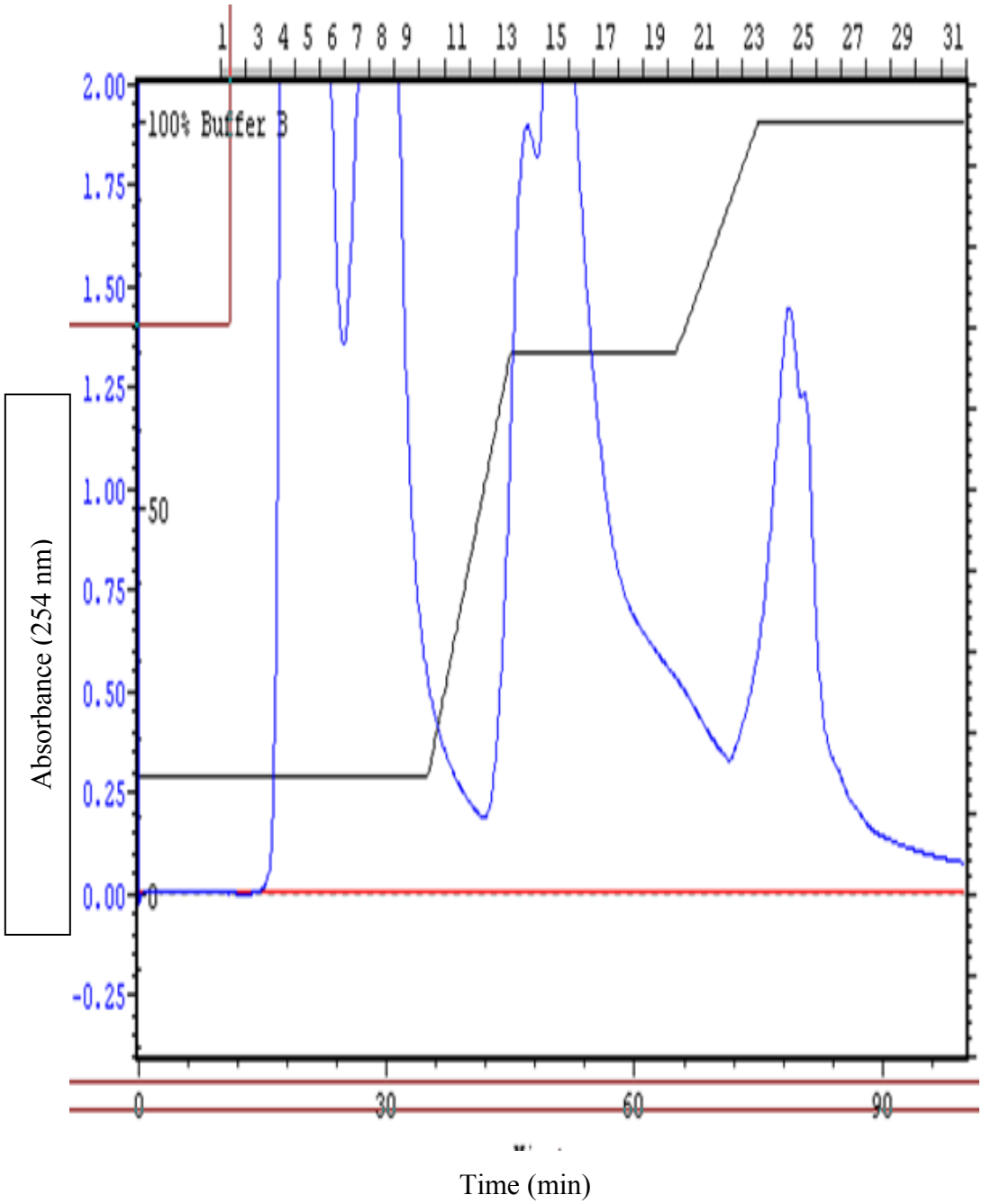


Figure 4. Example elution profile of reverse-phase C-18 flash chromatography of saponin-rich guar meal n-butanol extract. Fractions eluted at 20%, 60% and 100% methanol, respectively.

Isolation of 100% Methanol Sub-fractions

The 100% methanol extract was further separated into sub-fractions using preparative normal-phase high pressure liquid chromatography (Waters, model 510). In a small test tube 15 mg of freeze-dried the 100% methanol extract was dissolved in 1 mL of acetonitrile and 50-250 µl of the resultant solution was injected onto a silica gel preparative HPLC column (Waters, RCM 25 x 10). The column was preequilibrated and developed with isocratic acetonitrile:water (H_2O) + 0.025% trichloroacetic acid (TCA) (50:50, v:v) at a 4 mL/min flow rate using an isocratic mobile-phase over a 50 min run time. Four major peaks were detected at 254 nm (Waters 994, programmable photodiode array detector) and collected with a fraction collector (Gilson model 203) at 16, 39, 44, and 46 min retention times. This procedure was repeated to obtain sufficient purified material. Pooled material was evaporated under reduced pressure to remove the last traces of acetonitrile. A minimum volume of water was added to the dry fractions and the resulting material was freeze-dried, weighed and stored at room temperature until used in further studies.

An additional seven fractions of guar meal extracts were prepared by Dr. Mark Berhow (Agriculture Research Service, USDA, Peoria, IL). Guar meal was extracted nine times with methanol at 100^{o}C under 15, 000 psi in a Dionex ASE 300 automatic solvent extractor. The pooled extract was allowed to stand overnight and the residue was filtered and the extract dried at room temperature over several days. The dry extract was resuspended in water and partitioned with n-butanol and water, 1:1 (v: v) to collect n-butanol and aqueous solutions. The aqueous portion was partitioned with n-butanol two

more times to increase the yield of crude saponin in the n-butanol fraction. The pooled n-butanol extract was dried with a roto-evaporator. The dry n-butanol extract was resuspended in a mixture of water and methanol and loaded onto a C-18 stationary-phase flash chromatography column and eluted with an isocratic and then linear gradient of 30% to 80% acetonitrile against 0.2% acetic acid in water. Seven fractions (A, B, C, D, E, F and G) were detected by absorbance peaks at 210 nm and collected. A minimum volume of water was added and the fractions were freeze-dried until used in further studies.

Hemolytic Activity Assay

Chicken blood was collected from three mature roosters using 20 mL syringes with 18 gauge needles. Syringes were prefilled with 10 mL of phosphate buffered saline (PBS) supplemented with sodium citrate as anticoagulant (15.1 mg/mL PBS, 1:1, v/v). Blood samples were decanted into test tubes and placed in an ice bath. Red blood cells were separated by centrifugation (1,000 x *g* for 5 min) and resuspended with PBS. This procedure was repeated three additional times until the supernatant was colorless. A final 2% cell suspension of red blood cells was prepared by suspending 200 µL of red blood cells with 800 µL of PBS. Fresh cell suspensions were prepared daily.

Stock solutions of 2 mg of 20, 60, and 100% methanol saponin-rich guar meal extracts, four sub-fractions of the 100% methanol extract (16, 39, 43 and 46 min retention time) and seven fractions prepared by Dr. Mark Berhow were dissolved in 1 mL of PBS and filtered using 0.2-µm filters. The hemolytic assay was conducted in 96-

well flat bottom microtitre plates (eight rows by twelve columns) with low evaporation lids (Microtest™, Becton Dickinson, USA). One hundred microliters of PBS were added to all wells of the plate except the wells in the first two columns in which 100 µL of distilled water were added. Then, 100 µL of each 20, 60, and 100% methanol extract stock solutions were pipetted into the first row of columns 3 to 11 with three columns for each extract. The contents of the first row of wells in columns 3 to 11 were serially diluted through row 8 by aspirating and redispensing 3 times then transferring 100 µL to the next row. This procedure was repeated until 100 µL were discarded from each column after the last dilution. One hundred microliters of the 2% blood cell suspension were added to all wells of the plate. This procedure resulted in 8 dilutions ranging from 0.01-1.0 mg methanol extract/mL of PBS. The first two columns containing distilled water with blood were 100% hemolysis positive control wells, while column 12 containing PBS and blood alone served as the 0% hemolysis negative control wells. Each plate included three wells for each concentration of 20, 60, and 100% methanol extracts with two plates per replicate with three replicate plates per experiment.

The same procedure was used to assay the hemolytic activity of the four sub-fractions of the 100% methanol extract (16, 39, 43 and 46 min retention time), but with two microtiter columns for each sub-fraction and two columns for negative and positive controls. Each plate included two wells for each concentration of each fraction with two plates per replicate with three replicate plates per experiment.

Also, the same procedure was performed to assay the hemolytic activity of the seven fractions prepared by Dr. Mark Berhow using 50 µL rather than 100 µL of the 2%

blood cell suspension. Guar extract concentrations ranged from 0.005-0.666 mg/mL of PBS and each plate included one replicate for each concentration with two plates per replicate with three replicate plates per experiment.

Each plate was sealed with Parafilm (Pechiney Plastic Package, Neenah, WI) and covered with a polystyrene plate lid. Plates were incubated for 3 h at room temperature. Preliminary scanning of hemolysis in culture plate wells at wavelengths 405, 455, 520 and 650 nm showed that 650 nm was most efficient. The 650 nm wavelength allowed for optimum readings of blood cell lysis with little interference. Turbidity was measured by reading well optical density at 650 nm using a multi-well plate Bio-Rad® ELISA reader and observing a decrease in optical density associated with cell lysis.

Antibacterial Activity Assay

Minimum inhibitory concentration values (MIC) were used as a measure of antibacterial activity of 20, 60, and the 100% methanol saponin-rich guar meal extracts using two gram-positive bacteria, *Staphylococcus aureus* (ATCC 49525) and *Lactobucillus spp.* and two gram-negative bacteria, *Salmonella* Typhimurium (NCIM 2719) and *Escherichia coli* (ATCC 933). Average concentration of each bacterium was 1.90×10^6, 7.20×10^7, 5.74×10^5 and 3.88×10^6 colony forming units/mL (cfu/mL), respectively. Antibacterial activity of the four sub-fractions (16, 39, 43, and 46 min) of the 100% methanol extract were evaluated using one gram-positive bacterium, *Staphylococcus aureus* (ATCC 49525) and two gram-negative bacteria, *Salmonella* Typhimurium (NCIM 2719) and *Escherichia coli* (ATCC 933). Average concentration

of each bacterium was 1.90 x 10^6, 7.20 x 10^7 and 5.74 x10^5 colony forming units/mL (cfu/mL), respectively. The cfu/mL density of cultures was confirmed after distribution into assay plates by the following procedure. All bacterial stock solutions were serially diluted 10-fold and plated by inoculating 100 µL onto tryptic soy agar (TSA) (Difco™, Becton Dickinson, USA) plates. The plates were incubated for 24 h at 37°C. Colonies were counted to calculate the cfu/mL of the stock bacteria solutions. Dr. Berhow's 7 guar fractions were not evaluated for antibacterial activity due to insuffient quantity of guar extract.

Stock solutions of growing bacteria and standard stock concentrations of each extract were prepared. A stock solution of each bacterium was prepared by inoculating 100 µL of overnight cultures of each bacterium into 10 mL of sterile tryptic soy broth (TSB) (Bacto™, Becton Dickinson, USA) and incubating overnight at 37°C. Prior to use, all bacterial stock solutions were passed to new TSB every 8 h three times over a 24 h period to reach log phase growth for use in the study. A stock solution of each extract was prepared by dissolving 25 mg of each extract/mL of TSB and filtering solutions through 0.2-µm filters before use in 96-well plates. A stock solution of ampicillin was prepared at a concentration of 2 mg/mL of TSB as a positive control.

Minimum inhibitory concentration assays were conducted in 96-well flat bottom microtiter plates (eight rows by twelve columns) with low evaporation lids. One hundred microliters of TSB were added to all wells except the wells in the first column where 200-µL aliquots of TSB were added. The first column contained only TBS to assure absence of TSB contamination. Column 2 contained ampicillin as a positive control to

show minimal growth (0% bacterial growth) while columns 3-11 contained guar meal extracts with three columns for each extract. TSB with bacteria alone in column 12 served as a negative control for maximal growth (100% bacterial growth). This procedure resulted in eight serially 2-fold concentration dilutions ranging from 0.10-12.5 mg of each extract/mL of TSB, and 0.01-1.0 mg of ampicillin/mL of TSB as described previously. Each plate included three replicates of each extract at each concentration with two plates per replicate and three replicate plates per experiment.

The same procedure was used to assay the antibacterial activity of the four sub-fractions of the 100% methanol extract (16, 39, 43 and 46 min retention time), by adding 50 μL of each bacterium to each well. Methanol extract concentrations ranged from 0.005-0.666 mg/mL of TSB. Each plate included two wells for each concentration of guar fraction with two plates per replicate with three replicate plates per experiment. Preliminary scanning of bacterial growth in culture plate wells at wavelengths 405, 455, 520 and 650 nm showed that 650 nm was most efficient. The 650 nm wavelength allowed for optimum readings of bacterial growth with little interference in absorption by saponin extract solutions. Turbidity (growth or inhibition of the bacterial growth) was examined at 0 h and after 24 h of incubation using a Spectra Max 190 plate reader and Soft Max Pro. 3.0 Software (Molecular Devices Corp., Sunnyvale, CA). Bacterial growth was determined by comparison with negative controls in which 100% bacterial growth was recorded.

Statistical Analysis

Each treatment group resulted from 18 individual values (n = 3 replicates, two plates per replicate with 3 wells per plate) of 20, 60, 100% methanol saponin-rich guar meal extracts and 12 individual values (n = 3 replicates, two plates per replicate with 3 wells per plate) of four sub-fractions of the 100% methanol extract for the hemolytic and antibacterial activities, respectively. Dr. Berhow's seven fractions resulted in 6 individual values (n = 3 replicates, two plates per replicate with one well per plate) for hemolytic activity. Means of guar saponin fractions at each concentration were compared to the negative control group in order to establish significant differences among treatment groups in hemolytic and antibacterial activities. The MIC for each treatment was recorded at the lowest concentration which inhibited growth compared to the negative control group. Data obtained were subjected to one-way ANOVA and were expressed as mean ± standard error of mean (SEM) using the GLM program of statistical software (SPSS 14.0, SPSS Inc., Chicago, IL). Treatment means were separated ($P \leq 0.05$) using Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Initial guar meal ethanol extracts were subsequently partitioned by n-butanol and subjected to reverse-phase flash chromatography. This extract separated into a yellow and orange peak eluting with 20%, and 1 peak each with 60 and 100% methanol (Figure 4). Yields averaged 1.72 ± 0.47, 0.88 ± 0.16, 0.91 ± 0.16 and 1.55 ± 0.15% by weight of the original material, respectively. Further purification of the 100% methanol extract

using preparative normal-phase HPLC resulted in eluting 4 peaks at 16, 39, 44 and 46 min.

Saponins frequently are isolated by boiling in methanol (Massiot et al., 1991; Oleszek et al., 1992), ethanol (Levy et al., 1989; Oleszek et al., 1990) and n-butanol (Massiot et al., 1991; 1992). In this experiment, guar meal was extracted by ethanol:water (1:1, v:v) and the resultant extract was partionated with n-butanol (1:1, v:v) to elute crude guar saponin. Khalil and El-Adawy (1994) extracted saponins from peas, beans and soybean seeds by refluxing seed samples using four different methods. They extracted with ethanol:water (1:1) for 2.5 h in a water bath at 95^{o}C, pure methanol in a Soxhlet apparatus for 50 h, pure distilled water for 5 h in a boiling water bath, and phosphate buffered saline at pH 7.3 while shaking for 2 h. They found that the ethanol:water (1:1) extract showed the highest toxicity as assayed by hemolytic activity and fish mortality. Extraction with n-butanol efficiently isolates monodesmosidic and short-sugar-chain bisdesmosidic saponins, but results in partial or total loss of long-chain bisdesmosidic and trisdesmoside saponins (Oleszek, 1996).

Oleszek (1988) noted that saponin-rich plant extract purified using graded methanol concentrations showed that eluants up to 40% methanol remove carbohydrates and some phenolics. Eluants containing 50-60% methanol remove only the bisdesmoside saponins, while 70-80% methanol removes monondesmoside saponins. This phenomina may explain the antibacterial and hemolytic properties of the presumed saponin structures isolated by the 100% methanol solution rather than 20 and 60% methanol solutions reported later in this study. Curl et al. (1986) mentioned that the predominant

guar saponins have two sugar side chains, one attached at C-3 and another at C-29. It is not known whether the saponins isolated in the 100% methanol and 16-min sub-fractions are bidesmoside or monodesmoside saponins.

Further purification was performed using a 3-step methanol gradient in combination with a reverse-phase C-18 column and three methanol extracts (20, 60 and 100%) were eluted. Pure saponins can not usually be obtained by reverse-phase C-18 chromatography alone (Oleszek et al., 1990; 1992). Individual saponins can be selectively isolated from n-butanol extract subjected to reverse-phase C-18 chromatography followed by normal-phase HPLC silica gel column with water: acetonitrile solvent systems (Kesselmeier and Strack, 1981). In this experiment, further saponin purification was performed by subjecting the 100% methanol extract to normal-phase silica gel column, and four sub-fraction peaks were eluted at different retention times (16, 39, 43 and 46 min).

The 20 and 60% methanol extracts were not hemolytic at any concentration tested (Table 1). The water positive control (100% hemolysis) and PBS negative control (0% hemolysis) were used as standard references. The 100% methanol extract was hemolytic until diluted to less than 0.25 mg extract/mL. Further fractionation of the 100% methanol peak by HPLC yielded four retention time sub-fractions. The 16-min peak retention time was the only sub-fraction of the 100% methanol extract with hemolytic activity (Table 2). This fraction was hemolytic until diluted to less than 0.06 mg extract/mL of PBS concentration. Evaluation of the hemolytic activity of seven guar

Table 1. Hemolytic activity of saponin-rich guar meal extract separated into 20, 60 and 100% methanol fractions following reverse-phase C-18 chromatography

Saponin Extract	Positive Control[1]	Negative Control[2]	Methanol Extracts		
			20%	60%	100%
mg/mL	Optical Density (OD[3])				
1.00	0.16 ± 0.01^{c}	0.86 ± 0.05^{a}	0.74 ± 0.01^{a}	0.72 ± 0.01^{a}	0.39 ± 0.04^{b}
0.50	0.17 ± 0.01^{c}	0.81 ± 0.03^{a}	0.78 ± 0.01^{a}	0.77 ± 0.01^{a}	0.44 ± 0.04^{b}
0.25	0.15 ± 0.01^{c}	0.87 ± 0.04^{a}	0.78 ± 0.02^{a}	0.78 ± 0.02^{a}	0.54 ± 0.04^{b}
0.13	0.16 ± 0.02^{b}	0.84 ± 0.03^{a}	0.78 ± 0.01^{a}	0.79 ± 0.01^{a}	0.88 ± 0.02^{a}
0.06	0.18 ± 0.02^{b}	083 ± 0.08^{a}	0.79 ± 0.01^{a}	0.81 ± 0.02^{a}	0.86 ± 0.02^{a}
0.03	0.16 ± 0.01^{b}	0.85 ± 0.05^{a}	0.76 ± 0.01^{a}	0.80 ± 0.02^{a}	0.81 ± 0.01^{a}
0.02	0.16 ± 0.01^{b}	0.82 ± 0.01^{a}	0.77 ± 0.01^{a}	0.79 ± 0.01^{a}	0.81 ± 0.01^{a}
0.01	0.16 ± 0.01^{b}	0.85 ± 0.04^{a}	0.77 ± 0.01^{a}	0.76 ± 0.01^{a}	0.76 ± 0.01^{a}

[a-c]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Water replaced saponin solutions as a positive control since this treatment results in 100% hemolysis

[2]Phosphate buffered saline replaced saponin solutions as a negative control since this treatment results in 0% hemolysis.

[3]Lower values are associated with increased cell lysis.

Table 2. Hemolytic activity of four sub-fractions following normal-phase preparative HPLC of the 100% methanol fraction

Saponin Extract	Positive Control[1]	Negative Control[2]	Peak Retention Times (min) 16	39	44	46
mg/mL			Optical Density (OD[3])			
1.00	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.31 ± 0.01[b]	0.87 ± 0.01[a]	0.81 ± 0.01[a]	0.84 ± 0.04[a]
0.50	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.30 ± 0.01[b]	0.82 ± 0.01[a]	0.80 ± 0.01[a]	0.81 ± 0.04[a]
0.25	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.33 ± 0.01[b]	0.80 ± 0.02[a]	0.80 ± 0.02[a]	0.79 ± 0.04[a]
0.13	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.36 ± 0.01[b]	0.80 ± 0.01[a]	0.80 ± 0.01[a]	0.82 ± 0.02[a]
0.06	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.41 ± 0.01[b]	0.79 ± 0.01[a]	0.81 ± 0.02[a]	0.83 ± 0.02[a]
0.03	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.67 ± 0.01[a]	0.77 ± 0.01[a]	0.78 ± 0.02[a]	0.81 ± 0.01[a]
0.02	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.75 ± 0.01[a]	0.78 ± 0.01[a]	0.77 ± 0.01[a]	0.80 ± 0.01[a]
0.01	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.75 ± 0.01[a]	0.78 ± 0.01[a]	0.77 ± 0.01[a]	0.80 ± 0.01[a]

[a-b]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Water replaced saponin solutions as a positive control since this treatment results in 100% hemolysis

[2]Phosphate buffered saline replaced saponin solutions as a negative control since this treatment results in 0% hemolysis.

[3]Lower values are associated with increased cell lysis.

fractions prepared by Dr. Mark Berhow using reverse-phase C-18 chromatography indicated that the F and G fractions were the only two fractions having hemolytic activity until diluted to less than 0.042 and 0.666 mg extract/mL, respectively (Table 3). The F and G fractions were roughly equivalent to the 100% methanol extract eluted by our Texas A&M flash chromatography system. Fraction F was subjected to analytical HPLC (Figure 5) and at least 4 major peaks eluted with retention times between 30 and 40 min. Lindahl et al. (1957) reported that hemolysis can be used to identify the presence of saponins in plant extracts. However, hemolytic activity is not an attribute of all saponins. While quillaja saponins exhibit hemolytic activity (Jenkins and Atwal, 1994), other saponins from soybeans which are a mixture of soyasapogenol glycosides are not hemolytic (Gestetner et al., 1968). Also, not all saponins extracted from the same plant have the same hemolytic activity. Shany et al. (1970) showed that alfalfa root extracts are more hemolytic than those from alfalfa tops.

Some authors suggest that aglycone:glycone ratios (Segal et al., 1966; 1974), number of polar groups (Namba et al., 1973) and carbohydrate side chain length are the determinant features in hemolytic activity (Kuznetzova et al., 1982). Hemolytic activity is generally thought to be due to effects on cell membrane permeability by forming pores in membranes (Authi et al., 1988; El Izzi et al., 1992; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001), altering the sodium–potassium and calcium–magnesium ATPase activities (Choi et al., 2001), or insertion of the hydrophobic saponin nucleus into the lipid bilayer (Brain et al., 1990; Hu et al., 1996). Although we do not know the precise

Table 3. Hemolytic activity of seven fractions of guar meal eluted by flash reverse-phase C-18 chromatography

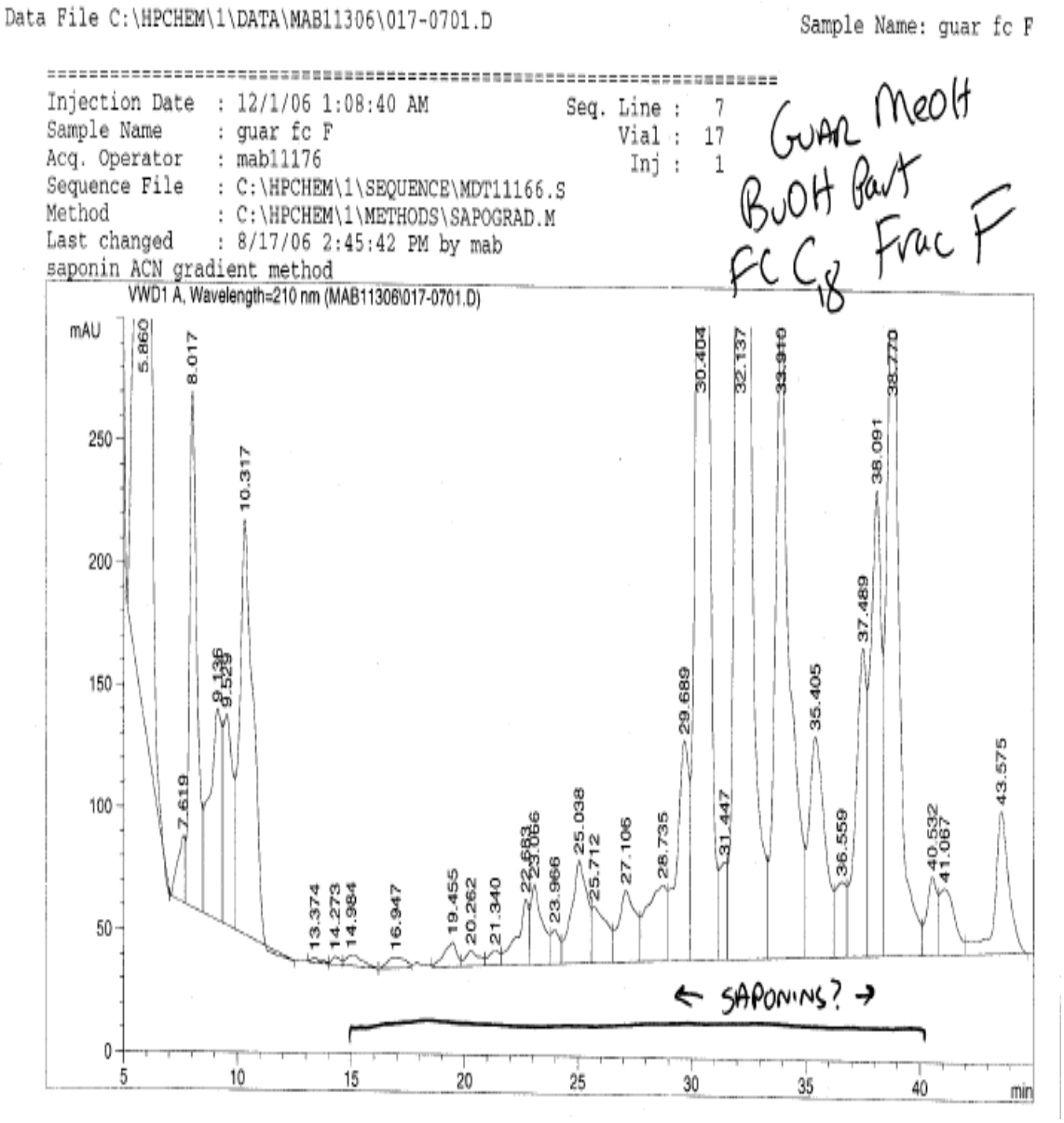
Saponin Extract	Positive Control[1]	Negative Control[2]	Fractions						
			A	B	C	D	E	F	G
mg/mL	Optical Density (OD[3])								
0.666	0.42 ± 0.01[c]	1.01 ± 0.05[a]	0.99 ± 0.01[a]	0.99 ± 0.01[a]	1.00 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	0.43 ± 0.01[c]	0.65 ± 0.01[b]
0.333	0.42 ± 0.01[c]	1.01 ± 0.05[a]	0.99 ± 0.01[a]	1.00 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	0.46 ± 0.01[b]	1.00 ± 0.01[a]
0.167	0.42 ± 0.01[b]	1.01 ± 0.05[a]	1.00 ± 0.01[a]	1.01 ± 0.02[a]	1.01 ± 0.02[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	0.50 ± 0.01[b]	1.00 ± 0.01[a]
0.083	0.42 ± 0.01[c]	1.01 ± 0.05[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	0.78 ± 0.01[b]	1.01 ± 0.01[a]
0.042	0.42 ± 0.01[b]	1.01 ± 0.05[a]	1.03 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.02[a]	1.01 ± 0.01[a]	1.02 ± 0.01[a]	0.99 ± 0.01[a]	1.01 ± 0.01[a]
0.021	0.42 ± 0.01[b]	1.01 ± 0.05[a]	1.03 ± 0.01[a]	1.02 ± 0.01[a]	1.02 ± 0.02[a]	1.02 ± 0.01[a]	1.01 ± 0.01[a]	1.00 ± 0.01[a]	1.02 ± 0.01[a]
0.011	0.42 ± 0.01[b]	1.01 ± 0.05[a]	1.02 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.00 ± 0.01[a]	1.02 ± 0.01[a]	1.01 ± 0.01[a]
0.005	0.42 ± 0.01[b]	1.01 ± 0.05[a]	1.02 ± 0.01[a]	1.02 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.01 ± 0.01[a]	1.02 ± 0.01[a]	1.02 ± 0.01[a]

[a-c]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Water replaced saponin solutions as a positive control since this treatment results in 100% hemolysis.

[2]Phosphate buffered saline replaced saponin solutions as a negative control since this treatment results in 0% hemolysis.

[3]Lower values are associated with increased cell lysis.



Retention time (min)

Figure 5. Example analytical HPLC chromatography of guar fraction (F) from Dr. Mark Berhow.

structure of the guar hemolytic fractions isolated in this study, researchers mention that the higher the R_f of saponins isolated, the higher the hemolytic activity using thin layer chromatography on silicia gel (Gestetner, 1971; Gestetner et al., 1971; Khalil and El-Adawy, 1994). High retention factors (R_f) are characteristics of less polar compounds as they travel with the solute as the mobile-phase on silicia gel plate or column (solid-phase or stationary-phase). These observations agree with the results obtained in this study where the 16-min peak had the highest R_f and the highest hemolytic activity among all sub-fractions of the 100% methanol extract.

The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic or, in this case, saponin-rich extract that can inhibit growth of a bacterium. The 100% methanol extract was more effective than either 20 or 60% methanol extracts against *Staphylococcus aureus, Lactobacillus spp*., *Escherichia coli* and *Salmonella* Typhimurium (Table 4).

It is of interest that the 20 and 60% methanol extracts showed a mild prebiotic effect with *Lactobacillus spp*. at concentrations ≥ 0.39 mg extract/mL. Minimum inhibitory concentrations were 3.13 and 0.78 mg extract/mL for 100% methanol saponin-rich guar meal extracts against gram-positive *Staphylococcus aureus* (Table 5) and *Lactobacillus spp*. (Table 6) respectively. A similar MIC was observed for the 100% extract exposed to the gram-negative *Escherichia coli* (Table 7) and *Salmonella* Typhimurium (Table 8) at 1.56 and 0.78 mg extract/mL, respectively.

The 16 min sub-fraction of the 100% methanol extract was the only effective fraction against *Staphylococcus aureus* at concentrations equal to or above than 0.333

mg extract/mL (Table 9). None of the sub-fractions of 100% methanol exhibited any antibacterial activity against both two gram-negative bacteria (*Escherichia coli* and *Salmonella* Typhimurium) at the concentrations tested in this study (Tables 10 and 11). The normal-phase sub-fractions were not tested against *Lactobacillus spp.* The results suggested some antibacterial activity was lost during the sub-fractioning of the reverse-phase 100% MeOH extraction.

Table 4. Minimal inhibitory concentration (MIC[1]) of antibacterial activity of reverse-phase 20, 60 and 100% methanol saponin-rich guar meal extracts against *Staphylococcus aureus*, *Lactobacillus spp*., *Escherichia coli* and *Salmonella* Typhimurium

Bacterium	Methanol Extract		
	100%	60%	20%
	MIC (mg/mL)		
Staphylococcus aureus	3.13	12.5	12.5
Lactobacillus spp.	0.78	PB[2]	PB
Escherichia coli	1.56	12.5	ND[3]
Salmonella Typhimurium	0.78	12.5	12.5

Means of MIC values of each saponin source against different bacteria show in the same column.
[1]MIC, the lowest concentration of antibiotic or saponin that inhibits growth of a microbe.
[2]PB, prebiotic effect detected as indicated by microbial growth stimulation at ≥ 0.39 mg/mL saponin extract.
[3]ND, MIC assay did not show antibacterial activity at the concentrations tested.

In general, many saponins are considered natural antimicrobial compounds making up a plant's defense systems (Mahato et al., 1982; Hussain and Cheeke, 1995;

Table 5. Antimicrobial activity against *Staphylococcus aureus* of reverse-phase 20, 60 and 100% methanol saponin-rich guar meal extracts

Saponin Extract	Positive Control[1]	Negative Control[2]	Methanol Extracts		
			100%	60%	20%
Mg/mL	Optical Density (OD[3])				
12.50	0.25 ± 0.05[d]	1.14 ± 0.012[a]	0.52 ± 0.01[c]	0.93 ± 0.03[b]	1.02 ± 0.03[b]
6.25	0.51 ± 0.01[c]	1.14 ± 0.012[a]	0.69 ± 0.02[b]	1.07 ± 0.04[a]	1.17 ± 0.02[a]
3.13	0.54 ± 0.12[c]	1.14 ± 0.012[a]	1.00 ± 0.03[b]	1.05 ± 0.04[a]	1.14 ± 0.02[a]
1.56	0.56 ± 0.13[b]	1.14 ± 0.012[a]	1.13 ± 0.02[a]	1.10 ± 0.04[a]	1.14 ± 0.03[a]
0.78	0.57 ± 0.13[b]	1.14 ± 0.012[a]	1.16 ± 0.02[a]	1.14 ± 0.03[a]	1.13 ± 0.02[a]
0.39	0.56 ± 0.13[b]	1.14 ± 0.012[a]	1.18 ± 0.01[a]	1.12 ± 0.03[a]	1.12 ± 0.02[a]
0.20	0.72 ± 0.08[b]	1.14 ± 0.012[a]	1.21 ± 0.02[a]	1.13 ± 0.02[a]	1.12 ± 0.02[a]
0.10	0.86 ± 0.04[a]	1.14 ± 0.012[a]	1.14 ± 0.01[a]	1.09 ± 0.02[a]	1.09 ± 0.02[a]

[a-d]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 6. Antimicrobial activity against *Lactobacillus spp*. of reverse-phase 20, 60 and 100% methanol saponin-rich guar meal extracts

Saponin Extract	Positive Control[1]	Negative Control[2]	Methanol Extracts 100%	60%	20%
mg/mL	Optical Density (OD[3])				
12.50	0.05 ± 0.01^{e}	1.25 ± 0.03^{c}	0.30 ± 0.03^{d}	1.42 ± 0.02^{a}	1.31 ± 0.01^{b}
6.25	0.05 ± 0.01^{e}	1.25 ± 0.03^{c}	0.77 ± 0.05^{d}	1.39 ± 0.01^{a}	1.35 ± 0.02^{b}
3.13	0.05 ± 0.01^{d}	1.25 ± 0.03^{b}	1.11 ± 0.01^{c}	1.32 ± 0.02^{a}	1.30 ± 0.02^{a}
1.56	0.05 ± 0.01^{d}	1.25 ± 0.03^{b}	1.07 ± 0.01^{c}	1.33 ± 0.02^{a}	1.31 ± 0.02^{a}
0.78	0.05 ± 0.01^{d}	1.25 ± 0.03^{b}	1.13 ± 0.02^{c}	1.34 ± 0.02^{a}	1.33 ± 0.02^{a}
0.39	0.26 ± 0.05^{c}	1.25 ± 0.03^{b}	1.23 ± 0.01^{b}	1.35 ± 0.03^{a}	1.30 ± 0.03^{a}
0.20	0.43 ± 0.07^{c}	1.25 ± 0.03^{b}	1.24 ± 0.01^{b}	1.34 ± 0.03^{a}	1.32 ± 0.02^{a}
0.10	0.70 ± 0.03^{b}	1.25 ± 0.03^{a}	1.20 ± 0.03^{a}	1.29 ± 0.04^{a}	1.25 ± 0.04^{a}

[a-e]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 7. Antimicrobial activity against *Escherichia coli* of reverse-phase 20, 60 and 100% methanol saponin-rich guar meal extracts

Saponin Extract	Positive Control[1]	Negative Control[2]	Methanol Extracts		
			100%	60%	20%
mg/mL	Optical Density (OD[3])				
12.50	0.05 ± 0.00^{d}	1.11 ± 0.02^{a}	0.43 ± 0.03^{c}	0.83 ± 0.04^{b}	1.14 ± 0.00^{a}
6.25	0.05 ± 0.01^{c}	1.11 ± 0.02^{a}	0.57 ± 0.02^{b}	1.14 ± 0.01^{a}	1.15 ± 0.01^{a}
3.13	0.06 ± 0.01^{c}	1.11 ± 0.02^{a}	0.82 ± 0.02^{b}	1.14 ± 0.02^{a}	1.13 ± 0.02^{a}
1.56	0.16 ± 0.01^{c}	1.11 ± 0.02^{a}	0.93 ± 0.01^{b}	1.14 ± 0.01^{a}	1.14 ± 0.01^{a}
0.78	0.38 ± 0.04^{b}	1.11 ± 0.02^{a}	1.00 ± 0.01^{a}	1.15 ± 0.01^{a}	1.11 ± 0.01^{a}
0.39	0.64 ± 0.03^{b}	1.11 ± 0.02^{a}	1.03 ± 0.01^{a}	1.13 ± 0.01^{a}	1.12 ± 0.01^{a}
0.20	0.81 ± 0.01^{b}	1.11 ± 0.02^{a}	1.06 ± 0.01^{a}	1.12 ± 0.01^{a}	1.11 ± 0.00^{a}
0.10	0.80 ± 0.01^{b}	1.11 ± 0.02^{a}	1.08 ± 0.03^{a}	1.16 ± 0.02^{a}	1.13 ± 0.00^{a}

[a-d]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 8. Antimicrobial activity against *Salmonella* Typhimurium of reverse-phase 20, 60 and 100% methanol saponin-rich guar meal extracts

Saponin Extract	Positive Control[1]	Negative Control[2]	Methanol Extracts		
			100%	60%	20%
mg/mL	Optical Density (OD[3])				
12.50	0.05 ± 0.00^{e}	1.20 ± 0.02^{a}	0.53 ± 0.02^{d}	0.94 ± 0.00^{c}	1.10 ± 0.01^{b}
6.25	0.05 ± 0.00^{c}	1.20 ± 0.02^{a}	0.67 ± 0.02^{b}	1.18 ± 0.00^{a}	1.21 ± 0.01^{a}
3.13	0.05 ± 0.01^{c}	1.20 ± 0.02^{a}	0.96 ± 0.02^{b}	1.16 ± 0.01^{a}	1.16 ± 0.01^{a}
1.56	0.06 ± 0.00^{c}	1.20 ± 0.02^{a}	1.04 ± 0.01^{b}	1.16 ± 0.01^{a}	1.15 ± 0.01^{a}
0.78	0.06 ± 0.00^{c}	1.20 ± 0.02^{a}	1.09 ± 0.01^{b}	1.19 ± 0.01^{a}	1.14 ± 0.00^{a}
0.39	0.15 ± 0.05^{b}	1.20 ± 0.02^{a}	1.10 ± 0.01^{a}	1.12 ± 0.02^{a}	1.13 ± 0.01^{a}
0.20	0.44 ± 0.07^{b}	1.20 ± 0.02^{a}	1.09 ± 0.01^{a}	1.14 ± 0.01^{a}	1.09 ± 0.04^{a}
0.10	1.18 ± 0.03^{a}	1.20 ± 0.02^{a}	1.10 ± 0.02^{a}	1.11 ± 0.02^{a}	1.12 ± 0.01^{a}

[a-e]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 9. Antimicrobial activity against *Staphylococcus aureus* of normal-phase 16, 39, 44 and 46 min sub-fractions of 100% methanol saponin-rich guar meal extract

Saponin Extract	Positive Control[1]	Negative Control[2]	Peak Retention Times (min)			
			16	39	44	46
mg/mL	Optical Density (OD[3])					
0.666	0.05 ± 0.01^{c}	0.51 ± 0.05^{a}	0.23 ± 0.01^{b}	0.54 ± 0.01^{a}	0.55 ± 0.01^{a}	0.55 ± 0.04^{a}
0.333	0.05 ± 0.01^{c}	0.51 ± 0.05^{a}	0.33 ± 0.01^{b}	0.46 ± 0.01^{a}	0.41 ± 0.01^{a}	0.42 ± 0.04^{a}
0.167	0.05 ± 0.01^{b}	0.51 ± 0.05^{a}	0.43 ± 0.01^{a}	0.43 ± 0.02^{a}	0.38 ± 0.02^{a}	0.38 ± 0.04^{a}
0.083	0.47 ± 0.01^{a}	0.51 ± 0.05^{a}	0.49 ± 0.01^{a}	0.42 ± 0.01^{a}	0.40 ± 0.01^{a}	0.40 ± 0.02^{a}
0.042	0.48 ± 0.01^{a}	0.51 ± 0.05^{a}	0.55 ± 0.01^{a}	0.38 ± 0.01^{a}	0.39 ± 0.02^{a}	0.40 ± 0.02^{a}
0.021	0.47 ± 0.01^{a}	0.51 ± 0.05^{a}	0.51 ± 0.01^{a}	0.40 ± 0.01^{a}	0.39 ± 0.02^{a}	0.39 ± 0.01^{a}
0.011	0.47 ± 0.01^{a}	0.51 ± 0.05^{a}	0.49 ± 0.01^{a}	0.40 ± 0.01^{a}	0.40 ± 0.01^{a}	0.41 ± 0.01^{a}
0.005	0.52 ± 0.01^{a}	0.51 ± 0.05^{a}	0.55 ± 0.01^{a}	0.46 ± 0.01^{a}	0.45 ± 0.01^{a}	0.45 ± 0.01^{a}

[a-c]Means (±SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.005 to 0.666 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 10. Antimicrobial activity against *Salmonella* Typhimurium of normal-phase 16, 39, 44 and 46 min sub-fractions of 100% methanol saponin-rich guar meal extract

Saponin Extract	Positive Control[1]	Negative Control[2]	Peak Retention Times (min)			
			16	39	44	46
mg/mL	Optical Density (OD[3])					
0.666	0.05 ± 0.01^{b}	1.01 ± 0.05^{a}	1.01 ± 0.01^{a}	1.02 ± 0.01^{a}	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}
0.333	0.05 ± 0.01^{b}	1.01 ± 0.05^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.00 ± 0.01^{a}
0.167	0.05 ± 0.01^{b}	1.01 ± 0.05^{a}	1.02 ± 0.01^{a}	1.00 ± 0.01^{a}	1.02 ± 0.01^{a}	1.01 ± 0.01^{a}
0.083	0.06 ± 0.01^{b}	1.01 ± 0.05^{a}	1.00 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}
0.042	0.09 ± 0.01^{b}	1.01 ± 0.05^{a}	1.01 ± 0.01^{a}	1.02 ± 0.01^{a}	1.01 ± 0.01^{a}	1.02 ± 0.01^{a}
0.021	0.28 ± 0.01^{b}	1.01 ± 0.05^{a}	1.02 ± 0.01^{a}	1.01 ± 0.01^{a}	1.02 ± 0.01^{a}	1.01 ± 0.01^{a}
0.011	0.55 ± 0.01^{b}	1.01 ± 0.05^{a}	1.01 ± 0.01^{a}	1.00 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}
0.005	0.83 ± 0.01^{b}	1.01 ± 0.05^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	1.02 ± 0.01^{a}

[a-c]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).
[1]Positive control, contained only ampicillin at 0.005 to 0.666 mg/mL.
[2]Negative control, contained neither ampicillin nor saponin.
[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 11. Antimicrobial activity against *Escherichia coli* of normal-phase 16, 39, 44 and 46 min sub-fractions of 100% methanol saponin-rich guar meal extract

Saponin Extract	Positive Control[1]	Negative Control[2]	Peak Retention Times (min)			
			16	39	44	46
mg/mL	Optical Density (OD[3])					
0.666	0.05 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}
0.333	0.05 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}
0.167	0.05 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.02^{a}	0.70 ± 0.02^{a}	0.71 ± 0.01^{a}
0.083	0.05 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.02^{a}
0.042	0.11 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.02^{a}	0.70 ± 0.02^{a}
0.021	0.29 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}	0.71 ± 0.02^{a}	0.71 ± 0.01^{a}
0.011	0.40 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.01^{a}	0.72 ± 0.01^{a}	0.71 ± 0.01^{a}
0.005	0.53 ± 0.01^{b}	0.72 ± 0.01^{a}	0.70 ± 0.01^{a}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}	0.71 ± 0.01^{a}

[a-b]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).
[1]Positive control, contained only ampicillin at 0.005 to 0.666 mg/mL.
[2]Negative control, contained neither ampicillin nor saponin.
[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Morrissey and Osbourn, 1999). Some studies reported antibacterial activity of dietary saponins in ruminant animals. For example, adding yucca extracts to the ruminant animal diet negatively affected cellulolytic bacteria without any effect on amylolytic bacteria (Wang et al., 2000a, b). In another study adding saponin-rich yucca powder or extract to ruminant animal diets inhibited gram-positive bacteria (Wallace et al., 1994; Cheeke, 1998; Cheeke and Otero, 2005). Also, some studies noted that quillaja saponin (*Quillaja saponaria*) and yucca saponin (*Yucca schidigera*) commercially produced by different companies showed different antibacterial activity against *Escherichia coli*, suggesting that saponins from various sources with different extraction procedures differ in their biological activities most likely due to different chemical structures (Sen et al., 1998b).

Kuete et al. (2006) reported significant antibacterial effects of saponin-rich methanolic extracts from the stem bark of *Tridesmostemon omphalocarpoides* (Sapotaceae). These observations agree with the results obtained from this study were the 100% methanol saponin-rich extract of guar meal exhibited antibacterial activity against all bacteria tested. Many saponins show a dose response effect on bacterial activity such as saponins isolated from *Bauhinia variegata* L. bark that exhibited more sensitivity for gram-negative bacteria than gram-positive bacteria at concentrations ranging from 2.5 to 10 mg/mL (Morrissey and Osbourn, 1999).

In contrast, our results showed that the saponin-rich extract from guar meal was more active against gram-positive bacteria than gram negative bacteria. These findings agree with the results obtained by several studies reporting that saponin showed

antibacterial activity against gram-positive bacteria more than gram-negative bacteria (Cioaca et al., 1978; Cheeke, 1998; Wallace et al., 1994; Cheeke and Otero, 2005; Avato et al., 2006). These differences may be due to the degradation of these saponins by some glucosidase enzymes produced by gram-negative bacteria. Variability of saponin glycone side chains in terms of number, chemical composition specific point of attachment to the steroid or triterpenoid nucleus is critical to the saponins biological effects (Cheeke, 1998; Osbourn, 2003).

The mode of action of antibacterial activity of saponins against both gram-negative and gram-positive bacteria is not yet clear. Avato et al. (2006) noted that the aglycone part of the saponin is the antibacterial determinant suggesting that the sugar moiety is not important for the antimicrobial efficacy while another study reported that saponins hydrolyzed by bacterial enzymes to its corresponding aglycone resulted in decreased antibacterial activity (Mandal et al., 2005).

Summary and Conclusions

Butanol extracts from guar meal yielded 4.8 ± 0.6% crude saponin by weight of the original material. Butanol extract purified by reverse-phase C-18 flash chromatography resulted in 2 peaks eluting with 20% methanol, and 1 peak each with 60, and 100% methanol to yield averages of 2.60 ± 0.32, 0.91 ± 0.16 and 1.55 ± 0.15% crude saponin by weight of the original material, respectively. Further normal-phase HPLC purification of the100% methanol saponin-rich guar meal extract eluted 4 peaks with 16, 39, 44 and 46 min retention times. Hemolytic activity was observed only in the

100 % methanol guar meal extract and 16 min sub-fraction of the methanol extract. Fractions F and G prepared by Dr. Mark Berhow also exhibited hemolytic activity. Antimicrobial activity of the 100% MeOH extract was most effective against *Staphylococcus aureus* and *Escherichia coli* bacteria. Only the 100% methanol extract exhibited both hemolytic and antibacterial activities against *Staphylococcus aureus*, *Lactobacillus spp*., *Salmonella* Typhimurium and *Escherichia coli*. The 20 and 60% methanol extracts were neither hemolytic nor antibacterially active, but stimulated *Lactobacillus spp*. Growth at concentrations more than or equal 0.39 mg/mL. Although one can presume the saponins are interacting in some way with the bacterial cell walls. The specific mode of action is not yet clear. So, more research is required to describe the mode of action of saponins against both gram-positive and gram-negative bacteria.

CHAPTER IV

ANTIMICROBIAL AND HEMOLYTIC ACTIVITIES DIFFER AMONG SAPONIN-RICH EXTRACTS FROM GUAR, QUILLAJA, YUCCA AND SOYBEAN

Introduction

Development of antimicrobial compounds remains an interesting and important area of research due to recent bans of a wide range of prophylactic antibiotics used in animal feed (Wallace, 2004). Concerns about bacterial resistance to antibiotics and subsequent restricted use of antibiotics for animal production applications have made development of the compounds a necessity. In the last decade, several antimicrobial like compounds have been suggested as antibiotic alternatives for prevention and treatment of pathogenic diseases, and to improve animal and poultry performance. These alternatives include dietary use of probiotics, prebiotics (Drake et al., 2003; Patterson and Burkholder, 2003; Murry et al., 2004; Higgins et al., 2005b; 2007; Tellez et al., 2006), organic acids (Van Immerseel et al., 2006), and medicinal herbs (Wink, 1999; Gordon and David, 2001; Du and Hu, 2004; Arab et al., 2006). Non-traditional treatments such as chlorate (Moore et al., 2006) and bacteriophages (Higgins et al., 2005a; Vicente et al., 2005) also have been recently evaluated. Most of the chemical structures of pharmaceutical compounds used today are structurally similar if not identical to compounds isolated from plant extracts (Du and Hu, 2004; Arab et al., 2006).

Among these original candidate chemical structures compounds are originating from plants such as quillaja, yucca, and soybean. So, these plants produce saponins which have several beneficial effects on animal health (Sen et al., 1998b; Avato et al., 2006). Guar, *Cyamopsis tetragonoloba* L. (syn. *C. psoraloides*) or cluster bean is a drought tolerant annual legume grown primarily for guar gum (galactomannan polysaccharide) that has many industrial and food processing applications. Guar meal is a combination of hull and germ fractions that is produced as a by-product of guar gum manufacture. Guar is also reported to contain between 5 and 13% curd triterpenoid saponin by weight of dry matter (Curl et al., 1986; Hassan et al., 2007) and thus contains potentially useful bioactive compounds.

Saponins are glycoside compounds composed of a fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) as in soybean, alfalfa, quillaja and guar, or alkaloid steroid (C-27) as in yucca, tomato and oats. One or more side chains of water-soluble sugars (glycone) are attached through ester linkages to the aglycone nucleus at different carbon sites (Tschesche et al., 1969; Kaneda et al., 1987; Haralampidis et al., 2002). Saponins have several biological effects, among them antibacterial (Mahato et al., 1982; Sen et al., 1998a, b), hemolytic (Schmidt-Thome and Prediger, 1950; Joos and Ruyssen, 1967; Seeman et al., 1973; Fukuda et al., 1985; Khalil and El-Adawy, 1994; Woldemichael and Wink, 2001) and anticancer (Ma et al., 2007) activities. Not all saponins have the same biological activities. While some saponins are beneficial, others are considered harmful to animal performance.

No research has evaluated the antibacterial and hemolytic activities of guar

saponin nor compared its biological activities with different saponin sources in a dose dependent manner. Therefore, this study was conducted to isolate saponin-rich guar meal extracts and to evaluate these extracts for hemolytic and antibacterial activities. A minimal inhibitory concentration (MIC) assay compared antibacterial activity of guar saponin extracts with three common commercial saponin sources (quillaja, yucca, and soybean) in a dose dependent manner.

Materials and Methods

Saponins

Commercial preparations of yucca extract containing 30% steroid saponin (Ultra Bio-Logic Inc. Rigadu, Quebec, Canada), quillaja extract containing 8.0-10% triterpenoid saponin (Sigma®, Saint Louis, MO, USA) and soybean extract containing 95% triterpenoid saponin (Organic Technologies, Coshocton, OH, USA) were purchased. Saponin-rich guar meal 100% MeOH extracts were prepared by refluxing guar meal with ethanol:water, 1:1 (v:v) then the resultant extract was partitioned with n-butanol:water, 1:1 (v:v). The n-butanol layer was freeze-drieded. Freeze-dried n-butanol extract was dissolved into distilled water and injected onto a preparative reverse-phase C-18 column and developed with a step gradient methanol:water (20:80, 60:40 and 100:0.00 (v:v), respectively) to elute three fractions (20, 60 and 100% methanol extracts) as previously described in chapter III.

Hemolytic Activity Assay

Hemolytic activity assays were performed as previously described in chapter III with some exceptions. A cell suspension of 1% chicken red blood cells was used and each treatment group resulted from 12 individual values (3 replicates of two plates per replicate with 2 columns per plate).

Antibacterial Activity Assay

Minimum inhibitory concentration (MIC) assays were used as a measure of antibacterial activity of saponin sources as previously described in chapter III with some exceptions. A single gram-positive bacterium (*Staphylococcus aureus* ATCC 49525) and two gram-negative bacteria (*Salmonella* Typhimurium, NCIM 2719; *Escherichia coli*, ATCC 933) were used. A stock solution of each saponin was prepared by dissolving 25 mg of saponin/mL of TSB and each treatment group resulted from 18 individual values (3 replicates of three plates per replicate with 2 columns per plate).

Statistical Analysis

Each treatment group resulted from 12 individual values (n = 3, three replicates of two plates per replicate with two wells per plate) for hemolytic activity and from 18 individual values (n = 3, three replicates of three plates per replicate with two wells per plate) for the antibacterial activity assay. The mean of a saponin fraction at each concentration was compared to the negative control group within a row to establish

significant differences among treatment groups in hemolytic and antibacterial activities. The MIC for each treatment was recorded at the lowest concentration which inhibited growth compared to the negative control group. Data obtained were subjected to one-way ANOVA and were expressed as mean ± standard error of mean (SEM) using the general linear model (GLM) program (SPSS 14.0, SPSS Inc., Chicago, IL). Treatment means were separated ($P \leq 0.05$) using Duncan's multiple range test (Duncan, 1955).

Results and Discussion

The 100% methanol extract yielded approximately 1.55 ± 0.15% by weight of the original guar meal extracted as described in chapter III. In this study the hemolytic and antibacterial activities of the 100% methanol extract were compared with three other common commercial saponin sources (quillaja, yucca, and soybean) in a dose dependent manner. The 20 and 60% methanol extracts were not tested as they were neither hemolytic nor antimicrobial as shown in chapter III. Oleszek (1988) noted that saponin-rich plant extract purified by graded methanol concentrations showed that eluants up to 40% methanol removed carbohydrates and some phenolics, eluants containing 50-60% methanol removed only the bisdesmoside saponins, while 70-80% methanol removed monondesmoside saponins. This phenomina may contribute to the antibacterial and hemolytic properties of the presumed saponin structures isolated by the 100% methanol extract rather than 20 and 60% methanol extracts reported in chapter III.

This study agrees with Choi et al. (2001), Menin et al. (2001) and Plock et al. (2001) who reported that not all saponins have hemolytic activity. The 100% methanol

saponin-rich guar meal extract and commericial quillaja saponin exhibited significantly higher hemolytic activity than both commericial yucca and soybean saponins at the same concentrations tested (Table 12). Commericial quillaja saponins were 100% hemolytic with substantial hemolysis occurring even at the lowest dose (0.01 mg/mL). The 100% methanol saponin-rich guar meal extract exhibited similar hemolytic activity until concentrations were ≤ 0.031 mg/mL. Yucca and soybean saponins showed hemolysis only at the highest concentration (1.0 mg/mL).

Hemolytic activity has been used by researchers to follow the isolation of saponins. It is the simplest and the fastest bioassay employed to detect and quantify some saponins in plant material (Authi et al., 1988; El Izzi et al., 1992; Khalil and EI-Adawy, 1994; Onning and Asp, 1995; Oleszek, 1996, Onning et al., 1996; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001).

Our results were in disagreement with Birk et al. (1963) and Birk and Peri (1980) who reported hemolytic activity of soybean saponins. Takechi and Tanaka (1995b) noted hemolytic rates of the steroid saponins were greater than the hemolytic rates of the triterpenoid saponins and Santos et al. (1997) reported that steroid saponins had higher hemolytic activity than triterpenoid saponins. On the other hand, our results agree with Jenkins and Atwal (1994) who reported hemolytic activity of quillaja, and Gestetner et al. (1968) who reported that soybean saponins, which are a mixture of soyasapogenol glycosides, were not hemolytic.

Table 12. Hemolytic activities of reverse-phase 100% methanol saponin-rich guar meal extract and solutions of saponins prepared from commercial extracts of quillaja, yucca and soybean

Saponin Extract	Positive Control[1]	Negative Control[2]	Saponin Extract Source			
			Guar Meal	Quillaja	Yucca	Soybean
mg/mL			Optical Density (OD[3])			
1.000	0.42 ± 0.01[c]	1.05 ± 0.02[a]	0.52 ± 0.02[c]	0.38 ± 0.04[c]	0.65 ± 0.07[b]	0.65 ± 0.02[b]
0.500	0.42 ± 0.01[b]	1.05 ± 0.02[a]	0.48 ± 0.01[b]	0.39 ± 0.05[b]	1.00 ± 0.02[a]	1.00 ± 0.01[a]
0.250	0.42 ± 0.01[b]	1.05 ± 0.02[a]	0.48 ± 0.01[b]	0.39 ± 0.06[b]	1.00 ± 0.02[a]	1.00 ± 0.03[a]
0.125	0.42 ± 0.01[b]	1.05 ± 0.02[a]	0.47 ± 0.01[b]	0.41 ± 0.06[b]	1.02 ± 0.03[a]	1.00 ± 0.03[a]
0.062	0.42 ± 0.01[b]	1.05 ± 0.02[a]	0.47 ± 0.01[b]	0.41 ± 0.06[b]	1.03 ± 0.03[a]	1.02 ± 0.03[a]
0.031	0.42 ± 0.01[b]	1.05 ± 0.02[a]	0.54 ± 0.01[b]	0.41 ± 0.07[b]	1.03 ± 0.03[a]	1.03 ± 0.03[a]
0.016	0.42 ± 0.01[b]	1.05 ± 0.02[a]	1.01 ± 0.01[a]	0.46 ± 0.07[b]	1.04 ± 0.03[a]	1.03 ± 0.04[a]
0.008	0.42 ± 0.01[c]	1.05 ± 0.02[a]	1.01 ± 0.02[a]	0.68 ± 0.07[b]	1.04 ± 0.04[a]	1.04 ± 0.03[a]

[a-c]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Water replaced saponin solution to serve as a positive control since this treatment results in 100% hemolysis.

[2]Phosphate buffered saline replaced saponin solution to serve as a negative control since this treatment results in 0% hemolysis.

[3]Lower values are associated with increased cell lysis.

Some research reported that hemolytic activity of saponins is attributed to the affinity of their aglycone moiety to sterols within membranes, particularly cholesterol (Schmidt-Thome and Prediger, 1950; Bangham et al., 1962; Glauert et al., 1962; Joosand Ruyssen 1967; Gee et al., 1998; Attele et al., 1999). Other research reported that hemolytic activity is decreased by removing the glycone moiety of the saponin (Santos et al., 1997), which is inconsistent with reports that the higher the hemolytic activity, the higher the ratio of aglycone:sugar (Shany et al., 1970; Gestetner et al., 1971).

Saponins possessing two or more sugar side chains show less hemolytic activity than saponins containing one sugar side chain (Fukuda et al., 1985; Mahato et al., 1988; Woldemichael and Wink, 2001). The monodesmoside saponins with glucose attached at C-3 show higher hemolytic activity than monodesmoside saponins with glucuronic acid at the same position (Oleszek, 1996).

The guar saponins tested in this study were not pure and their molecular structures are unknown at this time. Curl et al. (1986) reported that the primary saponin in guar was a bisdesmoside triterpenoid with a molecular weight of 1452. The stereochemistry of the saponin related to chain composition and length appears to be very important in conferring activity on the saponin molecule (Gee et al., 1998). As number of the monosaccharide units attached to the hydroxyl group at C-3 on the aglycone (Mahato et al., 1988) increases up to four to six sugar units, hemolytic activity increases (Anisimov et al., 1980; Kuznetzova et al., 1982). Also, hemolytic activity of saponins increases with decreasing numbers of polar groups on the aglycone moiety (Namba et al., 1973).

Gestetner et al. (1971) noted that alfalfa saponin, with the highest R_f value on silica gel showed the strongest hemolytic activity. The active groups on the aglycone and acylation of saponins also affect hemolytic activity. Neutral and acidic triterpenoids along with the acyl saponins are less hemolytic than the ester saponins (Gee et al., 1998; Attele et al., 1999; Oda et al., 2000). Laurence et al. (2005) noted that ethanol acylated triterpenoid saponins isolated from stem bark of *Harpullia austro-caledonica* showed 100% hemolysis of a 10% suspension of sheep erythrocytes. Deacylated quillaja saponins, which differ only in the absence of one glucose residue, showed decreased hemolytic activity (Pillion et al., 1996).

Some studies report that hemolytic activity is the result of saponin's effects on cell membrane permeability by either forming pores in membranes (Authi et al., 1988; El Izzi et al., 1992; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001), altering sodium–potassium, and calcium–magnesium ATPase activity (Choi et al., 2001), or insertion of the hydrophobic saponin nucleus into the lipid bilayer (Brain et al., 1990; Hu et al., 1996). Another study observed no direct relationship between the hemolysis and cell membrane permeability (Abe et al., 1978a). This is an unusual observation given hemolysis results in hemoglobin released from red blood cells.

The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic or saponin that can inhibit growth of a microbe. A general summary of MIC values for saponin extract solutions against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhimurium (Table 13) are compiled from more detailed data presented in Tables 14, 15, and 16. The 100% methanol guar meal extract and commericial quillaja

saponins were more effective than commericial yucca and soybean saponins which showed very weak or no antibacterial activity against any bacteria at the concentrations tested (Tables 13, 14, 15 and 16).

Minimum inhibitory concentrations against *Staphylococcus aureus* were 3.13, 0.10 and 12.5 mg/mL for the 100% methanol guar meal extract, commericial quillaja and yucca saponins, respectively (Tables 13 and 14). The MIC concentrations against *Salmonella* Typhimurium were 0.78 and 0.10 mg/mL for the 100% methanol guar meal extract and commercial quillaja saponins, respectively (Tables 13 and 15) and 1.56 and 0.10 mg/mL for the 100% methanol guar meal extract and commericial quillaja saponin against *Escherichia coli*, respectively (Tables 13 and 16). Commericial soybean saponin did not show antibacterial activity against *Staphylococcus aureus, Salmonella* Typhimurium and *Escherichia coli* while commericial yucca saponin did not show antibacterial activity against either *Salmonella* Typhimurium or *Escherichia coli* at any concentration tested in this study.

In general, many saponins are considered natural antimicrobial compounds that function as part of plant defense systems (Mahato et al., 1982; Hussain and Cheeke, 1995; Morrissey and Osbourn, 1999). Several studies reported antibacterial activity of dietary saponins in ruminant animals at different levels against different types of bacteria. For example, adding yucca extracts to a ruminant animal's diet negatively affected cellulolytic bacteria without any effect on amylolytic bacteria (Wang et al., 2000a, b). Also, yucca extract showed little or no antibacterial activity against gram-positive *Staphylococcus aureus* and *Lactobacillus spp.*, and gram-negative *Escherichia*

coli (Tanaka et al., 1996). In another study (Cheeke, 1998; Cheeke and Otero, 2005) adding saponin-rich yucca powder or extract to ruminant animals' diets showed more antimicrobial activity against gram-positive bacteria (*Staphylococcus aureus*) than gram-negative bacteria (*Escherichia coli*).

Table 13. Minimal inhibitory concentration (MIC[1]) of antibacterial activity of reverse-phase 100% methanol guar meal extract and solutions of saponins prepared from commericial extracts of quillaja, yucca and soybean on *Staphylococcus aureus*, *Salmonella* Typhimurium and *Escherichia coli* cultures

Bacterium	Saponin Extract Source			
	Guar Meal	Quillaja	Yucca	Soybean
	MIC (mg/mL)			
Staphylococcus aureus	3.13	0.10	12.5	ND[2]
Salmonella Typhimurium	0.78	0.10	ND	ND
Escherichia coli	1.56	0.10	ND	ND

Means of MIC values of each saponin source against different bacteria show in the same column.
[1]MIC, the lowest concentration of antibiotic or saponin inhibits growth of a microbe.
[2]ND, MIC assay did not show antibacterial activity at the concentrations tested.

Some saponins exhibit antimicrobial activity against gram-positive bacteria and no activity against gram-negative bacteria as in the yucca saponin tested in this study. The same observation was reported by Tanaka et al. (1996) who noted that the saponin fraction isolated from Soapnut pericarps (*Sapindus mukurossi*, which grows abundantly in China and Japan) showed moderate antibacterial activity against gram-positive bacteria, while no activity was observed against gram-negative bacteria. Conversely,

Table 14. Antimicrobial activities against *Staphylococcus aureus* of reverse-phase100% methanol guar meal extract and solutions of saponins prepared from commercial extracts of quillaja, yucca and soybean

Saponin Extract	Positive Control[1]	Negative Control[2]	Saponin Extract Source			
			Meal Guar	Quillaja	Yucca	Soybean
mg/mL	Optical Density (OD[3])					
12.5	0.25 ± 0.08^{d}	1.14 ± 0.01^{a}	0.51 ± 0.02^{c}	0.87 ± 0.05^{b}	0.88 ± 0.01^{b}	1.05 ± 0.04^{a}
6.25	0.51 ± 0.17^{e}	1.14 ± 0.01^{ab}	0.68 ± 0.03^{d}	0.97 ± 0.04^{c}	1.08 ± 0.01^{bc}	1.23 ± 0.02^{a}
3.13	0.54 ± 0.18^{c}	1.14 ± 0.01^{a}	0.98 ± 0.03^{b}	0.96 ± 0.05^{b}	1.11 ± 0.02^{ab}	1.19 ± 0.02^{a}
1.56	0.56 ± 0.19^{c}	1.14 ± 0.01^{a}	$1.12 \pm .03^{a}$	0.97 ± 0.05^{b}	1.15 ± 0.02^{a}	1.17 ± 0.02^{a}
0.78	0.57 ± 0.19^{c}	1.14 ± 0.01^{a}	1.15 ± 0.02^{a}	0.95 ± 0.05^{b}	1.15 ± 0.02^{a}	1.15 ± 0.02^{a}
0.39	0.56 ± 0.19^{c}	1.14 ± 0.01^{a}	1.18 ± 0.02^{a}	0.93 ± 0.05^{b}	1.13 ± 0.02^{a}	1.12 ± 0.02^{a}
0.20	0.72 ± 0.11^{c}	1.14 ± 0.01^{a}	1.21 ± 0.02^{a}	0.94 ± 0.05^{b}	1.14 ± 0.02^{a}	1.13 ± 0.02^{a}
0.10	0.86 ± 0.07^{b}	1.14 ± 0.01^{a}	1.13 ± 0.01^{a}	0.91 ± 0.05^{b}	1.11 ± 0.01^{a}	1.08 ± 0.02^{a}

[a-e] Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 15. Antimicrobial activities against *Salmonella* Typhimurium of reverse-phase 100% methanol guar meal extract and solutions of saponins prepared from commercial extracts of quillaja, yucca and soybean

Saponin Extract	Positive Control[1]	Negative Control[2]	Saponin Extract Source			
			Guar Meal	Quillaja	Yucca	Soybean
mg/mL	Optical Density (OD[3])					
12.5	0.05 ± 0.01^{d}	1.22 ± 0.02^{a}	0.54 ± 0.02^{c}	0.93 ± 0.01^{b}	0.92 ± 0.01^{b}	1.15 ± 0.02^{a}
6.25	0.05 ± 0.01^{d}	1.22 ± 0.02^{a}	0.68 ± 0.01^{c}	1.07 ± 0.02^{b}	1.10 ± 0.03^{b}	1.28 ± 0.01^{a}
3.13	0.05 ± 0.01^{d}	1.22 ± 0.02^{a}	0.98 ± 0.03^{c}	1.00 ± 0.01^{c}	1.10 ± 0.01^{b}	1.23 ± 0.01^{a}
1.56	0.06 ± 0.01^{d}	1.22 ± 0.02^{a}	1.05 ± 0.01^{c}	0.99 ± 0.02^{c}	1.15 ± 0.01^{b}	1.21 ± 0.01^{ab}
0.78	0.06 ± 0.01^{e}	1.22 ± 0.02^{a}	1.09 ± 0.01^{c}	1.00 ± 0.02^{d}	1.15 ± 0.01^{b}	1.18 ± 0.01^{ab}
0.39	0.15 ± 0.05^{c}	1.22 ± 0.02^{a}	1.10 ± 0.01^{a}	0.98 ± 0.02^{b}	1.15 ± 0.01^{a}	1.16 ± 0.01^{a}
0.20	0.44 ± 0.07^{c}	1.22 ± 0.02^{a}	1.10 ± 0.01^{ab}	0.97 ± 0.01^{b}	1.14 ± 0.01^{ab}	1.14 ± 0.01^{ab}
0.10	0.80 ± 0.03^{c}	1.22 ± 0.02^{a}	1.14 ± 0.02^{a}	0.94 ± 0.03^{b}	1.21 ± 0.02^{a}	1.18 ± 0.02^{a}

[a-e]Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, growth contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, growth contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Table 16. Antimicrobial activities against *Escherichia coli* of reverse-phase 100% methanol guar meal extract and solutions of saponins prepared from commercial extracts of quillaja, yucca and soybean

Saponin Extract	Positive Control[1]	Negative Control[2]	Saponin Extract Source			
			Guar Meal	Quillaja	Yucca	Soybean
mg/mL	Optical Density (OD[3])					
12.5	0.05 ± 0.01^{d}	1.11 ± 0.02 ^{a}	0.43 ± 0.03^{c}	0.97 ± 0.04^{b}	1.03 ± 0.02^{ab}	1.06 ± 0.02 ^{ab}
6.25	0.05 ± 0.01^{d}	1.11 ± 0.02 ^{a}	0.57 ± 0.02^{c}	1.04± 0.02^{b}	1.14 ± 0.01^{a}	1.14 ± 0.01^{a}
3.13	0.06 ± 0.01^{d}	1.11 ± 0.02 ^{a}	0.82 ± 0.02^{c}	0.94 ± 0.01^{b}	1.14 ± 0.01^{a}	1.13 ± 0.00^{a}
1.56	0.16 ± 0.02 ^{c}	1.11 ± 0.02 ^{a}	0.93 ± 0.01^{b}	0.92 ± 0.01 ^{b}	1.14 ± 0.01^{a}	1.12 ± 0.01^{a}
0.78	0.38 ± 0.04 ^{c}	1.11 ± 0.02 ^{a}	1.00 ± 0.01^{ab}	0.89 ± 0.01^{b}	1.12 ± 0.01^{a}	1.12 ± 0.01^{a}
0.39	0.64 ± 0.03 ^{c}	1.11 ± 0.02 ^{a}	1.03 ± 0.01^{a}	0.91 ± 0.02^{b}	1.09 ± 0.02^{a}	1.11 ± 0.01^{a}
0.20	0.81 ± 0.01^{c}	1.11 ± 0.02 ^{a}	1.06 ± 0.01^{a}	0.90 ± 0.02 ^{b}	1.08 ± 0.01^{a}	1.11 ± 0.01^{a}
0.10	0.80 ± 0.01^{b}	1.11 ± 0.02 ^{a}	1.08 ± 0.03^{a}	0.88 ± 0.01^{b}	1.04 ± 0.01^{a}	1.06 ± 0.01^{a}

$^{a-d}$Means (± SEM, n = 3) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

[1]Positive control, contained only ampicillin at 0.01 to 1.0 mg/mL.

[2]Negative control, contained neither ampicillin nor saponin.

[3]Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

saponins isolated from orchid tree (*Bauhinia variegata* L.) bark exhibited greater antibacterial activity for gram-negative bacteria than gram-positive bacteria at concentrations ranging from 2.5 to 10 mg/mL (Morrissey and Osbourn, 1999). Kuete et al. (2006) reported significant antibacterial effects of saponin-rich methanolic extracts from the stem bark of African *Tridesmostemon omphalocarpoides* locally known as "Babama".

Sen et al. (1998b) reported that commercially produced quillaja (*Quillaja saponaria*) and yucca (*Yucca schidigera*) saponins showed different antibacterial activities against *Escherichia coli*, suggesting that saponins from various commercial sources differ in their biological activities. This is most likely due to different extraction procedures which produce saponins with different chemical structures. In this study commercial quillaja and yucca saponins exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* at different concentrations. We did not evaluate different commercial sources of the same saponin however.

Saponin extraction procedures undoubtedly affect the antibacterial activity of the isolated saponins. Rakhimov et al. (1996) and Morrissey and Osbourn (1999) noted that fat free content extracts from the Hong Kong orchid tree (*Bauhinia variegata* L.) bark were more active than high fat extracts against gram-positive bacterial strains such as *Staphylococcus aureus.* Against gram-negative bacterial strains such as *Escherichia coli*, the fat free extracts exhibited either similar or less activity than the high fat extracts.

Results obtained in this study showed that the saponin-rich extract from guar meal was more active against gram-positive *Staphylococcus aureus* than gram-negative

Escherichia coli and *Salmonella* Typhimurium. These findings agree with the results obtained by several studies reporting that saponins showed antibacterial activity against gram-positive bacteria more than gram-negative bacteria (Cioaca et al., 1978; Cheeke, 1998; Wallace et al., 1994; Cheeke and Otero, 2005; Avato et al., 2006). This may be due to the degradation of saponins by glucosidase enzymes produced in higher concentrations by gram-negative bacteria than by gram-positive bacteria.

The MIC values of the 100% methanol extract were exactly the same as reported in chapter III against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium. The specific mode of action for the antibacterial activity of saponins against both gram-negative and gram-positive bacteria is not yet clear. One report suggests that the aglycone moiety of the saponin is the antibacterial determinant (Avato et al., 2006) and suggesting that the sugar moiety is not critical for antimicrobial efficacy while another study (Mandal et al., 2005) reported that saponins hydrolyzed by bacterial enzymes to its corresponding aglycone resulted in decreased antibacterial activity. As with hemolytic activity, the antibacterial activity of saponins likely is affected by many factors such as the aglycone, number, position and chemical structure of sugar side chains (Potter et al., 1993; Rakhimov et al., 1996). Guar, soybean and quillaja saponins are triterpenoid while yucca is a steroid saponin. The predominant guar saponin has two sugar side chains, one attached at C-3 and another at C-29 (Curl et al., 1986), while the predominant quillaja saponin (Higuchi et al., 1987; 1988) has two sugar side chains at C-3 and C-28. Yucca (Kaneda, et al., 1987; Cheeke et al., 2006) and soybean saponins (Kuduo et al., 1992) have two sugar side chains at C-3 and C-21. Yucca (Kaneda, et al.,

1987; Cheeke et at., 2006) and soyasaponin saponin (representing about 93% of the total soybean saponin) (Kuduo et al., 1992) have only one sugar side chain at C-3. While saponins may share similar sugar attachment sites, the sugar side chains themselves vary in type, number, sequence, and chain length. Quillaja saponin contains quillaic acid as the central aglycone attached with glucuronic acid, rhamnose, hexose and a fatty acyl chain in varying ratios (Higuchi et al., 1987), while soyasaponin I has a carboxylic group on the glucuronic acid aglycone (Oleszek, 1996).

Summary and Conclusions

The 100% methanol guar meal extract yielded approximately 1.55 ± 0.15% crude saponin by weight of the original guar meal dry matter. Saponin-rich extracts from different plant sources have different antibacterial and hemolytic activities. The 100% methanol guar meal extract and commericial quillaja saponins were significantly higher in both hemolytic and antibacterial activities against all bacteria tested than commercial yucca and soybean saponins at the same saponin concentration. Soybean saponin had no antibacterial activity against any of the bacteria at the saponin concentrations tested while yucca saponin had no antibacterial activity against the gram-negative bacteria at the concentrations tested. Hemolytic activity can be a predictor of antibacterial activity with different levels of some saponin-rich plant extracts such as guar and quillaja saponins while not for other saponins such as soybean and yucca saponins.

CHAPTER V

GUAR MEAL AMELIORATES EFFECTS OF *EIMERIA TENELLA* CHALLENGE IN BROILER CHICKS

Introduction

Avian coccidiosis (genus *Eimeria*) is a disease with an estimated $800 million worldwide economic impact for the poultry industry (Williams, 1998). Seven species of *Eimeria* infect chickens (*Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria necatrix, Eimeria praecox* and *Eimeria tenella*). Although all species are found in the USA poultry operations, *Eimeria acervulina, Eimeria maxima,* and *Eimeria tenella* are the most prevalent species. The incidence of detectable coccidiosis is as high as 50 to 70% on some poultry farms. This protozoan parasite exists in the form of a highly resistant oocyst in litter (Shirley, 1986).

Antiprotozoal chemical feed additives are the most widely used means of controlling genus *Eimeria* in chickens (Pogonka et al., 2003). While antiprotozoal "coccidiostats" effectively control avian coccidiosis, continuous use and misuse of anticoccidial drugs have led to the emergence of drug resistant strains (Long, 1982). Drug resistance in coccidial populations constantly threatens the continued usefulness of prophylactic chemotherapy. New coccidiostats must be continually developed to overcome increasing resistance to chemical drugs by the parasites (Chapman, 1993;

1998; Yadav and Gupta, 2001). Drug resistance and the high costs of developing novel drugs make discovery of alternative control methods beneficial (Chapman, 1997).

New products and husbandry strategies are needed to replace antibiotics or anticoccidial agents in poultry feeds. Antibiotic- or anticoccidia-resistant organisms have alarmed public health officials to possible loss of effective human disease treatments. In response, antibiotics or some anticoccidial agents used as growth promoters in poultry and animal feeds are banned or discouraged in many world markets (Nawaz et al., 2000; Bertolatti et al., 2003; Wallace, 2004). As this trend accelerates the search for plant-based antibacterial and anticoccidial compounds increases (Wink, 1999; Gordon and David, 2001; Murry et al., 2004; Van Immerseel et al., 2006). Traditional medicinal herbs sometimes are used to treat animal diseases (Du and Hu, 2004) and evidence toward protection of birds by using anticoccidial plant extracts (Arab et al., 2006) can be found.

Guar (*Gyamopsis tetragonoloba*) is a drought tolerant legume most prominently produced in India and Pakistan. The plant is grown for the guar bean's gum that has many industrial and food processing applications. Isolation of the galactomannan gum yields a high protein by-product containing guar hull, germ and residual gum. Guar meal also contains chemical compounds called saponins. Saponins are being currently investigated for antibacterial (Avato et al., 2006; Hassan et al., 2007), antiprotozoal (Mshvildadze et al., 2000), and antifungal (Yang et al., 2006) activities. Since both saponins and protein are concentrated in the hull and germ fractions of the guar bean, guar meal (Bakshi, 1966; Ambegaokar et al., 1969) may be useful as a practical

ingredient for poultry feed (Lee et al., 2003; a, b; 2005) which exhibit antiprotozoal activity against *Eimeria tenella*.

This study investigated the anticoccidial effects of guar meal in diets fed to chicks challenged with *Eimeria tenella*. Body weight, feed conversion ratio, mortality rate, duodenal pH, oocyst shedding per gram of feces, bloody diarrhea, and cecal lesion scores were measured.

Materials and Methods

A 2 x 2 factorial experimental design including 6 replicates of 5 chicks investigated the impact of 0 or 5% guar meal in corn-soy starter diets on chicks either unchallenged or challenged with *Eimeria tenella*. One hundred twenty, 1-d-old broiler chicks (Ross x Cobb) of mixed sex were obtained from a commercial hatchery and randomly distributed into wire battery cages. Diet formulas contained no anticoccidial feed additives (Table 17). Chicks were offered feed and water ad libitum, and were exposed to continuous light. Pooled feces were examined and collected from each replication. Oocyst shed, bloody feces score and cecal lesion score were determined.

Isolation, Sporulation and Counting of Oocysts

Sporulated oocysts of *Eimeria tenella* were obtained from Dr. David Caldwell's Laboratory, Departments of Poultry Science and Veterinary Pathobiology at College Station, Texas, USA. Infected chicks were challenged by 5,000 sporulated oocysts of *Eimeria tenella* in 0.5 mL of distilled water using crop gavage at 10 d of age.

Table 17. Composition[1] of broiler chicken starter diets containing either 0 or 5% guar meal

Ingredients	Guar Meal (%)	
	0	5
	(%)	
Corn	55.63	53.52
Guar meal[2]	0.00	5.00
Dehulled soybean meal	36.61	33.08
DL-Methionine	0.21	0.22
L-Lysine HCl	1.23	0.01
Fat (animal-vegetable blend)	3.81	4.43
Limestone	1.43	1.43
Mono-dicalcium PO_4	1.55	1.55
Salt	0.46	0.46
Trace minerals[3]	0.05	0.05
Vitamins[4]	0.25	0.25

[1] Calculated analysis of the diets was as follows: CP, 22.61%; ME, 3,100 kcal/kg; Ca, 0.90%; non-phytin P, 0.45%; methionine, 0.55%; lysine, 1.20%; threonine, 0.84%; and tryptophan, 0.27%.
[2] The nutrient matrix used was CP, 38.3%; ME, 2,033 kcal/kg Ca, 0.16% non-phytin P, 0.16% methionine, 0.45% lysine, 1.64% arginine, 4.90% threonine, 1.04% and tryptophan, 0.43%. Amino acid profile was by courtesy of Degussa Huls Corp., Allendale, NJ, 07401.
[3] Trace minerals premix added at this rate yields: 150 mg Mn, 16.80 mg Fe, 1.70 mg Cu, 125.50 mg Zn, 0.25 mg Se, 1.05 mg I, 0.84 mg Mo per kg diet.
[4] Vitamin premix added at this rate yields: 11,023 IU vitamin A, 46 IU vitamin E, 3,850 IU vitamin D_3, 1.47 mg vitamin k, 2.94 mg thiamine, 5.85 mg riboflavin, 20.21 mg pantothenic acid, 0.55 mg biotin, 1.75 mg folic acid, 477.67 mg choline, 16.50 µg vitamin B_{12}, 45.93 mg niacin, and 7.17 mg pyridoxine per kg diet.

Efficacy of guar meal against *Eimeria tenella* infection was evaluated by body weight, feed conversion ratio, mortality rate, duodenal pH, bloody diarrhea, oocysts shed per gram of feces, and cecal lesion score. Body weight and feed consumption were recorded weekly to determine feed conversion ratio in each treatment group from 0-3 wk of age. Mortality rate was calculated from the number of surviving chicks divided by the initial number of chicks. Degree of bloody diarrhea of each replication was assigned to one of five scores. Zero was normal status, and 1, 2, 3 and 4 corresponded to 1-25, 26-50, 51-75, or more than 75% of total feces containing blood, respectively (Youn and Noh, 2001).

Pooled fecal samples were collected daily from 6-10 d post challenge from each cage of the treatment groups. Progression of *Eimeria tenella* infections were monitored by the number of fecal oocysts shed per gram of feces using the McMaster counting technique (Dunn, 1969) as modified by Arslan et al. (2002). Chicks were euthanatized by cervical dislocation at 11 d post challenge (3 wk of age) to measure coccidial cecal lesion scores. Lesion scores were assigned to one of five ranks based on the epithelial color, fluid accumulation and the overall general appearance of the intestine (serosal thickness, mucosal erosion, dilation and similar factors) according to the method of Johnson and Reid (1970).

Statistical Analysis

Data obtained were subjected to one-way ANOVA and were expressed as mean ± standard error of mean (SEM) (SPSS 14.0, SPSS Inc., Chicago, IL). Treatment means were separated ($P \leq 0.05$) using Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Chicks challenged with *Eimeria tenella* shed oocysts in their feces (Table 18) while unchallenged chicks did not shed *Eimeria tenella* oocysts (data not shown). Feces containing coccidia oocysts is a route of reinnoculation and transmission between birds reared on the floor. In this study, challenged chicks were reared in batteries with wire flooring which limited rechallenge with *Eimeria tenella* sporulated oocysts.

Challenged chicks that were feed 5% guar meal significantly reduced the number of oocysts per gram shed in feces at 7 d and shed oocysts averaged over the entire 6-10 d post challenge collection period (Table 18). Oocyst output from challenged chicks decreased from 58.2×10^4 to 2.7×10^4 versus 5.52×10^4 to 0.5×10^4 from 7 to 10 d post challenge in those chicks fed 0 and 5% guar meal, respectively. Peak excretion of oocysts in challenged chicks fed 5% guar meal was delayed 1 d relative to challenged chicks fed 0% guar meal. Results of this study agree with Du and Hu (2004) who reported that peak extraction of fecal oocysts occurred at 7 and 8 d post infection. This result may be due to the antiprotozoal activity of guar meal saponin as reported in ruminant animals (Lu and Jorgensen, 1987; Wallace et al., 1994; Newbold et al., 1997; Cheeke et al., 2006). Presumably saponins exert antiprotozoal activity by binding with

sterol molecules present on protozoal cell membrane surfaces (Hussain and Cheeke, 1995).

Table 18. Effects of guar meal diet on oocyst per gram of feces shed from chickens challenged with *Eimeria tenella* at 10 d of age

	Guar Meal (%)	
Post Challenge	0	5
(d)	Oocysts Number ($x10^4$)	
6	0.79 ± 0.76^a	0.05 ± 0.03^a
7	58.21 ± 34.20^a	5.52 ± 2.20^b
8	18.70 ± 15.85^a	8.30 ± 3.12^a
9	1.14 ± 0.30^a	1.33 ± 0.32^a
10	2.71 ± 2.46^a	0.50 ± 0.20^a
$\sum$ 6-10	81.55 ± 45.91^a	15.69 ± 3.32^b

[a-b] Means (± SEM, n = 6) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).

Significant differences in cecal lesion scores, hemorrhages, severe bleeding and cores in cecal walls were observed between challenged chicks fed 0% guar meal and other treatment groups (Table 19). Bloody diarrhea was observed only in challenged chicks. Bloody diarrhea in challenged chicks fed 0% guar meal was observed only at 4-5 d post challenge with *Eimeria tenella* (Table 19). All chicks challenged with *Eimeria tenella* showed bloody droppings and a lack of appetite. These results agree with Hee and Jae (2001) who reported that bloody diarrhea occurs in chicks challenged with *Eimeria tenella* during 4-6 d post challenge.

Duodenal pH values for 0 and 5% guar meal fed chicks at 21 d of age did not differ between challenged (6.22 ± 0.05 versus 6.18 ± 0.06) and unchallenged (6.21 ±

0.84 versus 6.27 ± 0.09) treatments, respectively. No significant interaction was observed between the effects of *Eimeria tenella* challenge and body weight, body weight gain or feed conversion ratio. However, diet did significantly affect body weight, body weight gains and feed conversion ratio. No significant differences were observed in initial body weight among treatment groups (Table 20). Body weight and body weight gains of diet treatments were not significantly different at the start of the study and at 1 wk of age. At 2 wk of age, body weight and body weight gains of both challenged and unchallenged chicks fed 0% guar meal were significantly higher than those fed 5% guar meal. By 3 wk of age, body weight of challenged and unchallenged chicks fed 5% guar meal was lower than unchallenged chicks fed 0% guar meal. However, no significant

Table 19. Bloody feces score[1] recorded from 4 to 7 d post challenge in chicks unchallenged and challenged with *Eimeria tenella* and fed either a 0 or 5% guar meal diet

Post challenge	Unchallenged		Challenged	
	Guar Meal (%)		Guar Meal (%)	
	0	5	0	5
(d)				
4	0	0	2	0
5	0	0	3	0
6	0	0	0	0
7	0	0	0	0

[1] Results are expressed as pooled bloody feces where zero was normal status, and 1, 2, 3 and 4 corresponded to 1-25, 26-50, 51-75, or more than 75% of total feces containing blood, respectively (Youn and Noh, 2001). There were 30 chicks in each treatment group with 6 replicates of 5 chicks each.

differences in body weight between challenged chicks fed 0% guar meal and other treatment groups at 3 wk of age were observed. By 3 wk of age, body weight gains were not significantly different. The total of body weight gain over the entire experiment 1-3 wk of age of challenged and unchallenged chicks that were fed 5% guar meal was lower than unchallenged chicks fed 0% guar meal. However, no significant differences in body weight gain between challenged chicks fed 0% guar meal and other treatment groups at 3 wk of age (Table 20).

Unchallenged chicks that were fed either 0 or 5% guar meal showed significantly lower feed conversion ratio than challenged chicks that were fed 5% guar meal, but there were no significant differences between challenged chicks that were fed 0% guar meal and other treatment groups at 1 wk of age. Unchallenged chicks fed 0% guar meal showed significantly lower feed conversion ratio than unchallenged chicks fed 5% guar meal, but there were no significant differences in feed conversion ratio between challenged chicks fed either 0 or 5% guar meal and other treatment groups at two wk of age. No significant differences in feed conversion ratios among all treatment groups were observed at 3 wk of age. Cumulative feed conversion ratio over the entire experiment was significantly lower in unchallenged chicks fed 0% guar meal than unchallenged chicks that were fed 5% guar meal (Table 20). Mortality rate did not differ significantly among treatment groups. The amount of *Eimeria tenella* oocysts inoculated into chicks may have induced only a moderate challenge of *Eimeria tenella* that was of insufficient severity to cause mortality (Table 20). In the field, mortality caused by

Table 20. Weekly body weights, body weight gains, feed conversion ratio and mortality rate of chicks unchallenged or challenged with *Eimeria tenella* and fed either a 0 or 5% guar meal diet until 3 wk of age

Age	Unchallenged		Challenged	
	Guar Meal (%)		Guar Meal (%)	
	0	5	0	5
(wk)	Body weight (g)			
0	44.2 ± 0.8[a]	43.8 ± 0.5[a]	43.3 ± 0.7[a]	42.8 ± 0.7[a]
1	137.8 ± 6.6[a]	134.1 ± 2.9[a]	138.4 ± 4.0[a]	124.9 ± 5.0[a]
2	424.3 ± 8.2[a]	371.1 ± 14.0[b]	426.7 ± 9.4[a]	383.4 ± 5.8[b]
3	820.9 ± 14.4[a]	709.2 ± 28.7[b]	774.7 ± 19.4[ab]	746.3 ± 20.8[b]
	Body weight gains (g)			
1	93.63 ± 7.14[a]	90.25 ± 3.28[a]	95.07 ± 4.20[a]	82.08 ± 4.63[a]
2	286.46 ± 6.20[a]	237.07 ± 11.76[b]	288.33 ± 7.68[a]	258.59 ± 8.39[b]
3	396.63 ± 9.40[a]	338.09 ± 35.22[a]	347.93 ± 15.15[a]	362.90 ± 18.82[a]
∑ 0-3	776.72 ± 4.90[a]	665.42 ± 28.40[b]	731.33 ± 9.11[ab]	703.58 ± 21.06[b]
	Feed conversion ratio (g feed/g body gain)			
1	1.07 ± 0.07[b]	1.02 ± 0.07[b]	1.18 ± 0.03[ab]	1.28 ± 0.05[a]
2	1.24 ± 0.02[b]	1.34 ± 0.04[a]	1.27 ± 0.01[ab]	1.31 ± 0.03[ab]
3	1.52 ± 0.02[a]	1.83 ± 0.26[a]	1.69 ± 0.04[a]	1.62 ± 0.02[a]
∑ 0-3	1.36 ± 0.01[b]	1.50 ± 0.08[a]	1.46 ± 0.02[ab]	1.46 ± 0.01[ab]
	Mortality rate (%)			
1	0/30	1/30	1/30	1/30
2	0/30	0/29	0/29	1/29
3	0/30	0/29	0/29	0/28
∑ 0-3	0/30	1/30	1/30	2/30

[a-b] Means (± SEM, n = 6) within a row that do not share a common superscript are significantly different ($P \leq 0.05$). No significant difference of mortality was observed among treatment groups ($P \leq 0.05$).

Eimeria tenella can be as high as 50 % in poultry causing severe economic burdens (Williams, 1999).

These results agree with those of Kamran et al. (2002) who reported that adding 5% guar meal in chicken diets reduced body weight, feed efficiency and increased intestinal content viscosity. It appears the maximum percentage of guar meal appropriate for poultry diets is dependent on the bird's age. Some studies reported that there were no negative impacts on productive performance after adding guar meal without enzyme to diets at concentrations up to 2.5% in broiler chicks (Lee et al., 2003a, b) or 5% in laying hen diets (Gutierrez et al., 2007). Lee et al. (2005) reported that guar meal can be used at up to 5% with β-mannanase enzyme in broilers. High viscosity generally is coincident with delayed gastric emptying, increased small intestinal transit time, and inhibition of nutrient absorption (Blackburn and Johnson, 1981). Increased intestinal viscosity was proposed to result from residual guar gum present in guar meal. Guar meal used in poultry, pig and cattle rations is limited because of its adverse effects on feed intake, growth rate and feed efficiency (Brocher and Ackerson 1950; Saxena and Pradhan 1974; Thakur and Pradhan, 1975a, b). Growth is impacted by reduced nitrogen retention, slowed absorption of amino acids, reduced energy utilization and fat absorption (Anderson and Warnick, 1964; Katoch et al., 1971). Verma and McNab (1984a, b) reported that protein in guar meal was rich in arginine, but methionine and lysine concentrations were comparatively lower than concentrations typically found in soybean meal, and inadequate for optimum rat growth (Van Etten et al., 1961). Ambegaokar et al.

(1969) suggested that tryptophan, methionine and threonine were the first three deficient amino acids of guar meal when compared to whole egg protein.

Negative effects of adding 5% guar meal on body weight and feed conversion ratio in this study may be attributed to the presence of anti-nutrient compounds in guar meal such as trypsin inhibitor, excessive guar gum, saponin or some other unknown toxic substances. Conner (2002) proved that trypsin inhibitor activity in the raw guar meal was lower than trypsin inhibitor levels found in heat-treated soybean meal. Therefore, trypsin inhibitor is not likely the primary anti-nutritive factor in guar. A series of feeding experiments conducted by Vohra and Kratzer (1964) demonstrated that as little as 1% guar gum in broiler chicken diets causes a depression of growth. When the diet contained 2% guar gum, the relative growth of broiler chickens was 61-67% of controls. Lee et al. (2003a) also supported the idea that residual guar gum was at least partially responsible for the effects seen when guar meal was fed.

The growth depressing properties of guar gum may be overcome by treating the feed with enzymes capable of hydrolyzing it, namely pectinase, cellulose, a preparation from sprouted guar beans (Vohra and Kratzer, 1964) or β-mannanase (Vohra and Kratzer, 1964; 1965; Chesson, 1987; Lee et al., 2003a, b). It is thought that feed containing exogenous enzymes with guar meal reduced intestinal viscosity and alleviated the deleterious effects associated with residual guar gum.

Guar meal also is rich in saponins (Curl et al., 1986; Hassan et al., 2007) which have antinutritional properties (Price et al., 1987; Mahato et al., 1988; Shimoyamada et al., 1990). Saponins can be harmful for monogastric animals such as swine, fish and

poultry (Newman et al., 1958; Sim et al., 1984; Terapunduwat and Tasaki, 1986; Potter et al., 1993; Jenkins and Atwal, 1994; Olli et al., 1994; Francis et al., 2001a, b). They tend to have a bitter taste, irritate the membranes of the mouth and the digestive tract (Oleszek and Jurzysta, 1992; Oleszek et al., 1994), reduce intestinal motility (Klita et al., 1996), change ingesta transit time (Ueda et al., 1996), inhibit gastric emptying (Matsuda et al., 1999a, b; Yoshikawa et al., 2001) and decrease growth rate (Ueda and Ohshima, 1987; Cheeke, 1983; 1996; Makkar and Becker, 1996). They also lower digestion rate (Freeland et al., 1985; Lu and Jorgensen, 1987; Lu et al., 1987; Killeen et al., 1998), reduce protein digestibility (Potter et al., 1993; Shimoyamada et al., 1998), depress mucosal enzyme activity in the lower intestine (Olli et al., 1994), perhaps as a stressor stimulate ACTH and corticosterone levels (Kim et al., 1999), inhibit the absorption of vitamins A and E in chicks and iron in the rat (Johnson et al., 1986; Southon et al., 1988a; Jenkins and Atwal, 1994). Interestingly they have been reported to increase mineral excretion of sodium, iron, magnesium and zinc (West, 1979; Freeland et al., 1985; Southon et al., 1988b), as well as change the circadian rhythm of feeding behavior (Petit et al., 1995).

On the other hand, other studies reported no adverse effect on growth after feeding fenugreek seeds containing steroid saponins (Ishaaya et al., 1969) and soybean triterpenoid saponins (Petit et al., 1995) at concentrations as much as five times the concentration in a normal soybean-supplemented diet of chicks, rats and mice. Yucca and quillaja saponins are classified as generally recognized as safe (GRAS) and are approved for food use in the USA (Fenwick et al., 1992). It was not clear which specific

compounds in guar meal contributes to the anticoccidial or growth inhibitory effects. The most likely candidates are residual guar gum and triterpenoid saponins however.

Summary and Conclusions

Chicks were successfully challenged with *Eimeria tenella* and a mild infection was created as determined by fecal shedding of oocysts and bloody feces. The infection was mild and resulted in no increased mortality perhaps because of the low initial dose of *Eimeria tenella* and the fact that the chicks were grown on wire floors that avoids reinfection. Addition of 5% guar meal in the diet decreased the severity and perhaps delayed the *Eimeria tenella* infection since oocyst's shed in the feces were greatly reduced and the peak day of shedding was delayed. Results indicated that adding 5% guar meal to the diet of chicks challenged with *Eimeria tenella* reduced infection as measured by oocyst output in the feces and bloody diarrhea. Further research is needed to differentiate whether the anticoccicdiosis effect of guar meal was due to residual guar gum (glacactomannans), saponin, or some unknown component of guar meal.

CHAPTER VI

ANTIBACTERIAL ACTIVITIES OF GUAR MEAL, GUAR GUM AND SAPONIN-RICH GUAR MEAL EXTRACTS AGAINST *CLOSTRIDIUM PERFRINGENS* IN CHICKENS

Introduction

Clostridium perfringens is a gram-positive, spore-forming anaerobic bacterium that is a normal inhabitant of the gut microflora and may be found in the crop, gizzard, small intestine and ceca of many animals including chickens (Johansson and Sarles, 1948; Shapiro and Sarles, 1949; Smith, 1965; Willis, 1977; Komnenov et al., 1981; Gazdzinski and Julian, 1992; Branton et al., 1997). Processed poultry meat also has relatively high numbers of *Clostridium perfringens* (Craven et al., 2001b) with transmission to humans through the consumption of poultry products (Craven et al., 2001a, b). *Clostridium perfringens* also occurs widely in the environment, including water, soil, and workers clothing and boots (Willis, 1977). However, the presence of *Clostridium perfringens* per se, does not lead directly to disease. Although 75-95% of birds are colonized by *Clostridium perfringens*, only a small proportion of these birds ever show symptoms of the disease (Miwa et al., 1997; Craven et al., 2001a, b). *Clostridium perfringens* produces many extracellular enzymes and toxins including alpha (α), theta (τ) and kappa (κ) toxins (Rood, 1998) in addition to epsilon (ε) endotoxin which is activated upon exposure to trypsin and chymotrypsin, and is associated with B and D strains of clostridia (Petit et al., 2001). All strain types (A-E) of

the bacterium contain the α-toxin gene (McDonel, 1986; Songer, 1996; Petit et al., 2001).

Clostridium perfringens, the causative organism of necrotic enteritis is often found in relatively small numbers, usually less than 10^4 colony forming units (cfu) in the gastrointestinal tract of most bird species (Asaoka et al., 2004). Necrotic enteritis is an enteric disease that affects a variety of bird species and increases mortality rate in broiler chickens (Cowen et al., 1987), laying hens (Broussard et al., 1986), turkeys (Gazdzinski and Julian, 1992; Droual et al., 1995) and quail (Berkhoff, 1985). Necrotic enteritis in poultry is associated with α and β-toxin producing *Clostridium perfringens* strains type A or C (Van Immerseel et al., 2004). The clostridial β-toxin induces hemorrhagic necrosis of the intestinal mucosa (Baba et al., 1992). Necrotic enteritis is an acute, infectious and non contagious disease (Schwartz, 1988) affecting chickens 2-24 wk of age (Long, 1973; Ficken, 1991; Ross, 1999). Upon examination of the gastrointestinal tract and associated organs of affected birds, gross lesions are found, usually in the jejunum and ileum. The proximate small intestine and ceca show less evidence of lesions, but may be distended by gas and bloody fluids (Frame and Bickford, 1986). The intestinal mucosa may be covered with a layer of fibrino-necrotic material (Porter, 1998).

Necrotic enteritis disease also can be divided into clinical and subclinical necrotic enteritis categories. Clinical signs of necrotic enteritis include depression, decreased appetite, decreased digestion, diarrhea, and severe necrosis of the intestinal tract leading ultimately to mortality (Kaldhusdal and Hofshagen, 1992; Ficken and

Wages, 1997; Porter 1998; Kaldhusdal and Lovland, 2000; Lovland and Kaldhusdal, 2001). The subclinical form of the disease causes a decrease in overall performance of birds and has been associated with hepatic lesions (Lovland and Kaldhusdal, 1999). The incidence of necrotic enteritis varies from 1-40% of commercial broiler flocks in North America and the European Union (Kaldhusdal and Skjerve, 1996; Kaldhusdal and Lovland, 2000; Annett et al., 2002). Necrotic enteritis is a disease in poultry that can have high economic and animal welfare costs which have become increasingly prevalent in the European Union due to factors such as the removal of antibiotic growth promoters and the animal by-products from diet formulations. In 2000, the subclinical form of necrotic enteritis cost producers as much as five cents per bird due to decreased performance in the USA (Van der Sluis, 2000). Cost of this disease, including clinical and subclinical infections, was close to $2 billion dollars worldwide.

Reducing the effects of *Clostridium perfringens* in experimental settings has been evaluated with a variety of management tools, including use of antibiotics, vaccines, and competitive exclusion cultures (Hofacre et al., 1998; Williams et al., 2003; Lovland et al., 2004). Currently, antibiotic growth promoters predominantly are used in the commercial poultry settings and improve the health and performance of poultry (Bedford, 2000). *Clostridium perfringens* is a bacterium specifically targeted by antibiotic growth promoters. Many of these products, such as avoparcin, adriamycin, bacitracin, virginiamycin, and tylosin, have been removed from production practices in the European Union (Van Immerseel et al., 2004).

There are many plant containing antibacterial compounds, among them guar saponins (Livingston et al., 1977; Avato et al., 2006; Hassan et al., 2007). Guar meal is a by-product produced by isolating the guar gum from guar bean (*Gyamopsis tetragonoloba*). Guar meal contains 33-47.5% crude protein (Bakshi, 1966; Ambegaokar et al., 1969) and can be used at low concentrations in poultry feeds (Lee et al., 2003a, b; Lee et al., 2005). The meal contains 13-18% guar gum (Bakshi et al., 1964; Lee et al., 2004) and 5-13% crude saponin by weight of the dry matter (Curl et al., 1986; Hassan et al., 2007). This study was conducted to investigate whether addition of either 2.5% guar meal, or 1% guar gum or 0.125% saponin-rich guar meal extract in a 55% wheat corn/soy based starter broiler diet would ameliorate lesions of necrotic enteritis in broiler chicks challenged with *Clostridium perfringens*.

Materials and Methods

Isolation of Saponin-Rich Guar Extract

Guar meal (Rhodia Inc., Vernon, TX) was ground in a commericial coffee mill to form a fine powder and subsequently extracted by refluxing 25 g with 250 mL of ethanol:water, 1:1 (v:v) for 3 h in a simple reflux apparatus. Refluxed extracts were cooled and filtered through 150-µm (Watman No. 2) then 125-µm pore size (Watman No. 114) filter papers. Ethanol was removed from the filtrate by evaporating under reduced pressure in a roto-evaporator (Buchi, Rinco Instrument Co., Inc., Greenville, IL, Switzerland, model 310391) until two-thirds of the initial volume was removed. The

remaining aqueous extract was partitioned with n-butanol, 1:1 (v: v) overnight at room temperature using a separatory funnel. The upper n-butanol extract was collected in a glass flask and the lower aqueous extract was further partitioned with n-butanol two more times to increase the yield of crude saponin. The butanol extracts were pooled and evaporated to dryness using the roto-evaporator procedure described above. A minimum volume of water was added to the dry n-butanol extract and the resulting crude saponin was freeze-dried, weighed and stored at room temperature until used in further studies.

Experimental Chicks

Three hundred one-d-old unsexed Ross x Cobb broiler chicks were purchased from a local commercial hatchery and placed on clean pine shaving litter. Birds were reared in 2.4 x 1.2 m pens, allowing 0.12 m^2 of pen space per bird. Water and feed were provided ad libitum with 22 light:2 dark h schedules throughout the study. All treatment groups were fed isocalloric and nitrogenous diets containing 3050 metabolizable energy and 22.6% CP (Table 21).

Experimental Design

Chicks were randomly distributed among five treatments with three replicates of 20 chicks per replicate. Birds were assigned to one of the following treatments: 1) negative control (NC), chicks fed broiler starter diet containing 55% wheat but unchallenged with *Clostridium perfringens* type A, 2) positive control, chicks fed broiler

Table 21. Composition of a control[1] (C) starter broiler chicken diet and diets containing either 2.5% guar meal[2] (GM), 1.0% guar gum (GG), or 0.125% saponin-rich guar meal extract (GS)

	Dietary Treatments			
Ingredients (%)	C	GM	GG	GS
Corn	5.35	5.00	5.00	5.09
Wheat, Hard	55.00	55.00	55.00	55.00
Saponin-rich guar meal extract	0.00	0.00	0.00	1.25
Guar gum	0.00	0.00	1.00	0.00
Guar meal[2]	0.00	2.50	0.00	0.00
Soybean meal (48.5%, CP)	28.80	26.14	27.05	28.85
DL-Methionine	0.25	0.27	0.28	0.25
L-Lysine HCl	0.29	0.68	1.20	0.29
Fat (animal-vegetable blend)	6.26	6.34	6.39	6.34
Limestone	1.63	1.63	1.63	1.63
Mono-dicalcium PO_4	1.72	1.73	1.74	1.72
Salt	0.41	0.41	0.41	0.41
Trace minerals[3]	0.05	0.05	0.05	0.05
Vitamins[4]	0.25	0.25	0.25	0.25

[1]Calculated analysis of the diets was as follows: CP, 22.6%; ME, 3,050 kcal/kg; Ca, 1.00%; non-phytin P, 0.50%; methionine, 0.57%; lysine, 1.30%; threonine, 0.77%; tryptophan, 0.28%.

[2]The nutrient matrix used was CP, 38.3%; ME, 2,030 kcal/kg; Ca, 0.16%; non-phytin P, 0.16%; methionine, 0.45%; lysine, 1.64%; arginine, 4.90%; threonine, 1.04%; and tryptophan 0.43%. Amino acid profile was by courtesy of Degussa Huls Corp., Allendale, NJ, 07401.

[3]Trace minerals premix added at this rate yields: 150 mg Mn, 16.80 mg Fe, 1.70 mg Cu, 125.50 mg Zn, 0.25 mg Se, 1.05 mg I, 0.84 mg Mo per kg diet.

[4]Vitamin premix added at this rate yields: 11, 02 IU vitamin A, 46 IU vitamin E, 3, 85 IU vitamin D_3, 1.47 mg Vitamin k, 2.94 mg thiamine, 5.85 mg riboflavin, 20.21 mg pantothenic acid, 0.55 mg biotin, 1.75 mg folic acid, 477.67 mg choline, 16.50 µg vitamin B_{12}, 45.93 mg niacin, and 7.17 mg pyridoxine per kg diet.

starter diet containing 55% wheat and challenged with *Clostridium perfringens* type A, 3) chicks fed broiler starter diet containing 55% wheat and 2.5% guar meal and challenged with *Clostridium perfringens* type A, 4) chicks fed broiler starter diet containing 55% wheat and 1% guar gum and challenged with *Clostridium perfringens* type A, and 5) chicks fed broiler starter diet containing 55% wheat and 0.125% saponin-rich guar meal extract and challenged with *Clostridium perfringens* type A.

Preparation and Administration of *Clostridium perfringens*

Four field isolates of *Clostridium perfringens* (type A) from different geographical locations (1 isolate each from Texas and Virginia, and 2 isolates from Georgia) were cultured separately then combined and provided to the appropriate treatment groups as described by McReynolds et al. (2004). For challenge, the isolates were grown in thioglycollate broth media in an anaerobic chamber for 12 h at 37^{o}C. Chicks were challenged by oral crop gavage with 10^{7} cfu of *Clostridium perfringens*/0.5 mL once daily for 3 consecutive d starting at d 14. Weekly body weight, feed intake and mortality rate were recorded from 0-21 d of age. The weak or unhealthy chicks were weighted and excluded before chicks were challenged at 14 d of age to avoid any other interaction factors. At 21 d of age, necropsies were performed and intestinal lesions were scored for each chick of all the treatment groups.

Necrotic Enteritis Lesion Scores

The jejunum and ileum of the small intestine were examined to evaluate gross lesions associated with necrotic enteritis. All chickens were euthanatized by cervical dislocation and necropsied at the termination of the experiment (21d of age). The intestine of each chicken was excised cut longitudinally about 15-20 cm in length from the pars pyloris gastris (below the gizzard) to Meckel's diverticulum was removed to determine the lesion scores. A sample of gastrointestinal contents was collected for determination of *Clostridium perfringens* numbers in digesta. Lesion scores were recorded using the following criteria: 0 = no gross lesions, normal intestinal appearance; 1 = thin-walled or friable, gray appearance; 2 = thin-walled, focal necrosis, gray appearance, small amounts of gas production; 3 = thin-walled, sizable patches of necrosis, gas-filled intestine, small flecks of blood; and 4 = severe extensive necrosis, marked hemorrhage, much gas in intestine according to Prescott (1979).

Colony Forming Unit (cfu) Concentrations

To quantitatively measure populations of *Clostridium perfringens* the sample of gastrointestinal contents collected from excised intestines was diluted and plated in an anaerobic hood. The gastrointestinal contents (1g) from excised small intestines of each chick were placed into 10 mL of anaerobic thioglycollate broth media and stomached for 30 s. Half a mL of the resultant gut contents was removed from the stomached material, placed into an anaerobic vial containing 4.5 mL of anaerobic thioglycollate broth media placed into an anaerobic chamber and incubated for 18 h at 40°C. Next, ten-fold 6 serial

dilutions were applied and 100 µL of each dilution was inoculated on Shahidi-Ferguson-perfringens (SFP) agar plates and anaerobically incubated at 40°C for 18 h. The plates were examined for the presence of *Clostridium perfringens* and the colony forming units (cfu) exhibiting *Clostridium perfringens* morphology are counted.

Statistical Analysis

The attempt to induce necrotic enteritis in the experiment was unsuccessful as evidenced by absence of disease lesions and absence of difference between negative and positive controls. Therefore, the data obtained were subjected to one-way ANOVA and were expressed as mean ± standard error of mean (SEM) using the GLM procedure of a statistical software package (SPSS 14.0, SPSS Inc., Chicago, IL). Treatment means were separated ($P \leq 0.05$) using Duncan's multiple range test (Duncan, 1955).

Results and Disscusion

Challenge by *Clostridium perfringens* in this study was unsuccessful and did not result in necrotic enteritis as evidenced by the absence of significant differences between the negative and positive control groups in lesion score or *Clostridium perfringens* digesta colony forming units (Table 22). Feed intake, feed conversion ratio, mortality rate (Table 23), body weight and body weight gain (Table 24) also were not different between these two treatments.

Several factors are thought to induce necrotic enteritis such as diet composition (Smith, 1965; Nairn and Bamford, 1967), high dietary levels of fish meal (Johnson and

Pinedo, 1971; Truscott and Al-Sheikhly, 1977; Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992; Kocher et al., 2003), high dietary levels of wheat and barley (Branton et al., 1987; Riddell and Kong, 1992), increased stocking density and poor litter quality (Al-Sheikhly and Truscott, 1977), previous diseases such as *Eimeria tenella* (Frame and Bickford, 1986), birds' age (Long, 1973; Ficken, 1991; Ross, 1999), season (Kaldhusdal and Skjerve, 1996) and increased digesta viscosity (Kocher et al., 2003).

In this study we used diets containing a high percentage of wheat (55%) and oral doses of *Clostridium perfringens* to induce the necrotic enteritis according to Branton et al. (1997) and McReynolds et al. (2004). They reported that necrotic enteritis lesion scores were lower for chicks challenged with *Clostridium perfringens* and fed corn-based diets than those chicks challenged with *Clostridium perfringens* and fed wheat-corn-based diets. Perhaps necrotic enteritis was not observed among treatments because only one dose of *Clostridium perfringens* was innoculated (10^7 cfu/mL once daily for 3 consecutive days starting on d 14). McReynolds et al. (2004) observed necrotic enteritis lesions after challenging chicks with *Clostridium perfringens* with 10^7 cfu/mL twice daily for 3 consecutive days starting on d 17. Helmboldt and Bryant (1971) noted that low challenge dose of the *Clostridium perfringens* is non-pathogenic.

The positive control treatment and the guar meal, guar gum and saponin-rich guar meal extract dietary treatments were all given oral doses of *Clostridium perfringens*. Since no differences were observed between positive and negative controls,

the only meaningful comparisons for the effects of guar component dietary treatments are with the positive control.

The number of *Clostridium perfringens* colony forming units of chicks fed 1% guar gum was significantly higher than positive control chicks. However, cfu number of guar meal and saponin-rich extract dietary treatments were not different than the cfu counts of the guar gum dietary treatment (Table 22).

Although Lalitha et al. (1990) reported increased mortality after orally using 300 mg of yucca steroid saponin/kg body weight in rats, no mortality differences were observed in our study. Not even differences in feed intake were observed among the dietary treatments (Table 23). There are many incidents of feed intake suppression by alfalfa triterpenoid saponins (Sim et al., 1984; Terapunduwat and Tasaki, 1986; Potter et al., 1993; Jenkins and Atwal, 1994). Conversely, the feed intake and appetite of Wister rats improved when they were fed 10 and 100 mg/300 g body weight of saponin-rich extract from fenugreek seed which contains steroid saponins (Petit et al., 1993). Results also disagree with studies that report reduced feed intake in poultry, pig and cattle fed rations containing guar meal (Brocher and Ackerson 1950; Saxena and Pradhan 1974; Thakur and Pradhan, 1975a, b). These differences may be attributed to different chemical structures and concentrations of the saponins fed, and the concentrations of guar meal and gum formulated in the diets.

Diet significantly affected feed conversion ratio (Table 23), body weight and body weight gain (Table 24) however. Significant differences in the feed conversion

Table 22. Duodenal lesion scores[1] and colony forming units (cfu) of duodenal contents from chicks unchallenged (NC) or challenged with *Clostridium perfringens* and fed a positive control (PC), 2.5% guar meal GM), 1% guar gum (GG), or 0.125% saponin-rich guar meal extract (GS) diet

Parameters	Treatments				
	NC[2]	PC[3]	GM[4]	GG[5]	GS[6]
Lesion Score	1.3 ± 0.1[a]	1.3 ± 0.1[a]	1.6 ± 0.1 [a]	1.3 ± 0.1[a]	1.5 ± 0.1[a]
(n)	(36)	(32)	(38)	(38)	(41)
Colony Forming Units (cfu/mL x 10^{-4})	8.2 ± 5.1[b]	25.3 ± 13.0[b]	42.8 ± 32.9[ab]	125.1 ± 37.0[a]	90.2 ± 44.3[ab]
(n)	(15)	(15)	(15)	(15)	(15)

[a-b]Means (± SEM) within a row that do not share a common superscript are significantly different (≤ 0.05).
[1]Lesion scores were recorded using the following criteria (Prescott, 1979): 0 = no gross lesions, normal intestinal appearance; 1 = thin-walled or friable, gray appearance; 2 = thin-walled, focal necrosis, gray appearance, small amounts of gas production; 3 = thin-walled, sizable patches of necrosis, gas-filled intestine, small flecks of blood; and 4 = severe extensive necrosis, marked hemorrhage, much gas in intestine.
[2]NC, 55% wheat-corn-based starter broiler diet unchallenged with *Clostridium perfringens*.
[3]PC, 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[4]GM, 2.5% added to 55% wheat-corn-based starter broiler challenged with *Clostridium perfringens*.
[5]GG, 1.0% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[6]GS, 0.125% added to 55% wheat-corn-based starter broiler diet challenged with C*lostridium perfringens*.

Table 23. Feed intake, feed conversion ratio and mortality rate for chicks unchallenged (NC) or challenged with *Clostridium perfringens* and fed a positive control (PC), 2.5% guar meal (GM), 1% guar gum (GG), or 0.125% saponin-rich guar meal extract (GS) diet

Age	Treatments				
	NC[1]	PC[2]	GM[3]	GG[4]	GS[5]
(d)			Feed intake (g)		
1-7	115.0 ± 8.2[a]	1.22.8 ± 4.9[a]	125.1 ± 1.7[a]	113.5 ± 5.3[a]	115.5 ± 2.8[a]
8-14	243.2 ± 10.6[a]	244.6 ± 7.8[a]	263.0 ± 15.9[a]	221.2 ± 11.8[a]	236.2 ± 18.9[a]
15-21	466.9 ± 31.7[a]	478.3 ± 29.5[a]	427.1 ± 51.8[a]	431.2 ± 20.5[a]	426.8 ± 31.8[a]
			Feed conversion ratio (g feed/g body gain)		
1-7	1.20 ± 0.08[a]	1.33 ± 0.09[a]	1.28 ± 0.03[a]	1.23 ± 0.01[a]	1.22 ± 0.02[a]
8-14	1.08 ± 0.05[a]	1.18 ± 0.08[a]	1.13 ± 0.03[a]	1.10 ± 0.09[a]	1.18 ± 0.05[a]
15-21	1.23 ± 0.08[b]	1.12 ± 0.09[b]	1.49 ± 0.06[ab]	1.34 ± 0.09[ab]	1.63 ± 0.17[a]
			Mortality rate (%)		
0-14	10.0±2.9[a]	15.0±7.6[a]	8.3±6.0[a]	13.3±7.3[a]	6.7±3.3[a]
15-21	18.5±6.5[a]	21.6±0.4[a]	24.0±13.3[a]	25.3±10.1[a]	14.1±7.1[a]

[a-b]Means (± SEM) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).
[1]NC, 55% wheat-corn-based starter broiler diet unchallenged with *Clostridium perfringens*.
[2]PC, 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[3]GM, 2.5% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[4]GG, 1.0% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[5]GS, 0.125% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.

Table 24. Weekly body weight and body weight gain for chicks unchallenged (NC) or challenged with *Clostridium perfringens* and fed a positive control (PC), 2.5% guar meal (GM), 1% guar gum (GG), or 0.125% saponin-rich guar meal extract (GS) diet

Age	Treatments				
	NC[1]	PC[2]	GM[3]	GG[4]	GS[5]
(d)	Body Weight (g)				
0	43.3 ± 0.4[a]	44.5 ± 0.6[a]	43.8 ± 0.5[a]	45.4 ± 0.7[a]	44.6 ± 0.2[a]
7	38.7 ± 7.0[a]	137.3 ± 2.4[a]	141.6 ± 2.8[a]	136.4 ± 4.0[a]	138.9 ± 3.4[a]
14	366.3 ± 5.7[a]	346.3 ± 15.1[a]	373.1 ±12.4[a]	358.6 ± 18.1[a]	341.0 ± 27.3[a]
21	746.2 ± 22.7[a]	748.7 ± 32.1[a]	659.4 ± 22.3[b]	682.6 ± 11.7[ab]	612.7 ± 21.7[b]
	Body Gain Weight (g)				
0-7	96.4 ± 6.6[a]	92.9 ± 2.9[a]	97.8 ± 2.3[a]	92.1 ± 4.2[a]	94.3 ± 3.4[a]
8-14	226.6 ± 9.5[a]	209.0 ± 13.3[a]	231.5 ± 9.7[a]	222.1 ± 20.8[a]	202.1 ± 23.9[a]
15-21	380.0 ± 22.6[a]	402.4 ± 42.6[a]	286.3 ± 31.9[b]	324.0 ± 9.5[ab]	271.7 ± 49.0[b]

[a-b]Means (± SEM) within a row that do not share a common superscript are significantly different ($P \leq 0.05$).
[1]NC, 55% wheat-corn-based starter broiler diet unchallenged with *Clostridium perfringens*.
[2]PC, 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[3]GM, 2.5% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[4]GG, 1.0% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.
[5]GS, 0.125% added to 55% wheat-corn-based starter broiler diet challenged with *Clostridium perfringens*.

ratio were only observed from 15 to 21 d of age between both negative and positive control groups and chicks fed 0.125% saponin-rich guar meal extract. Guar meal extract increased the feed conversion ratio above control levels, but were not different from 2.5% guar meal and 1% guar gum fed chicks (Table 23).

The only dietary effects on body weight and body weight gain were observed during the last week of the study (Table 24). Body weights of chicks fed guar meal and saponin-rich extract diets were depressed in comparison to control chicks at 21 d, but were not depressed in comparison with chicks fed guar gum. Body weight gains were depressed below those of control chicks during 15 to 21 days by guar meal and saponin-rich extract diets (Table 24). These results agree with researchers who reported that guar meal used in poultry, pig and cattle rations is limited because of its adverse effects on growth rate (Brocher and Ackerson 1950; Saxena and Pradhan 1974; Thakur and Pradhan, 1975a, b). Kamran et al. (2002) reported that adding 5% guar meal in chicken diets reduced body weight and intestinal content viscosity. Our results disagree with the observations of Lee et al. (2003a, b) who reported that there were no negative impacts on productive performance after adding guar meal without enzyme to diets at concentrations up to 2.5% in broiler chicks. Lee et al. (2005) reported that guar meal can be used at up to 5% with β-mannanase enzyme in broilers. High viscosity generally is coincident with delayed gastric emptying, increased small intestinal transit time, and inhibition of nutrient absorption (Blackburn and Johnson, 1981). Increased intestinal viscosity was proposed to result from residual guar gum present in guar meal.

Growth is impacted by reduced nitrogen retention, slowed absorption of amino acids, reduced energy utilization and fat absorption (Anderson and Warnick, 1964; Katoch et al., 1971). Verma and McNab (1984a, b) reported that protein in guar meal was rich in arginine, but methionine and lysine concentrations were comparatively lower than concentrations typically found in soybean meal, and inadequate for optimum rat growth (Van Etten et al., 1961). Ambegaokar et al. (1969) suggested that tryptophan, methionine and threonine were the first three deficient amino acids of guar meal when compared to whole egg protein.

Previous studies reported that the negative effects of adding guar meal on body weight and feed conversion ratio might be attributed to the presence of anti-nutrient compounds in guar meal such as trypsin inhibitor, excessive guar gum, saponin or some other unknown toxic substances. Guar meal used in poultry ration was limited by its adverse effects on growth rate (Thakur and Pradhan, 1975a, b) due its saponin and guar gum contents. Guar meal contains 5-13% of dry matter triterpenoid guar saponin (Curl et al., 1986; Hassan et al., 2007) and 13-18% guar gum, residual galactomannan gum (Bakshi et al., 1964; Lee et al., 2004). Conner (2002) proved that trypsin inhibitor activity in the raw guar meal was lower than trypsin inhibitor levels found in heat-treated soybean meal. Therefore, trypsin inhibitor is not likely the primary anti-nutritive factor in guar. Our results agree with observations of Vohra and Kratzer (1964) who demonstrated that as little as 1% guar gum in broiler chicken diets causes a depression of growth. When the diet contained 2% guar gum, the relative growth of broiler chickens

was 61-67% of controls. Lee et al. (2003a) also supported the idea that residual guar gum was at least partially responsible for the effects seen when guar meal was fed.

The growth depressing properties of guar gum may be overcome by treating the feed with enzymes capable of hydrolyzing it, namely pectinase, cellulose, a preparation from sprouted guar beans (Vohra and Kratzer, 1964) or β-mannanase (Vohra and Kratzer, 1964; 1965; Chesson, 1987; Lee et al., 2003a, b). It is thought that feed containing exogenous enzymes with guar meal reduced intestinal viscosity and alleviated the deleterious effects associated with residual guar gum.

Guar meal also is rich in saponins (Curl et al., 1986; Hassan et al., 2007) which may have antinutritional properties such as those of alfalfa saponins (Price et al., 1987; Mahato et al., 1988; Shimoyamada et al., 1990). Saponins can be harmful for monogastric animals such as swine, fish and poultry (Newman et al., 1958; Sim et al., 1984; Terapunduwat and Tasaki, 1986; Potter et al., 1993; Jenkins and Atwal, 1994; Olli et al., 1994; Francis et al., 2001a, b). They tend to have a bitter taste, irritate the membranes of the mouth and the digestive tract (Oleszek and Jurzysta, 1992; Oleszek et al., 1994), reduce intestinal motility (Klita et al., 1996), change ingesta transit time (Ueda et al., 1996), inhibit gastric emptying (Matsuda et al., 1999a, b; Yoshikawa et al., 2001) and decrease growth rate (Ueda and Ohshima, 1987; Cheeke, 1983; 1996; Makkar and Becker, 1996). They also lower digestion rate (Freeland et al., 1985; Lu and Jorgensen, 1987; Lu et al., 1987; Killeen et al., 1998), reduce protein digestibility (Potter et al., 1993; Shimoyamada et al., 1998), depress mucosal enzyme activity in the lower intestine (Olli et al., 1994). Perhaps acting as a stressor, they stimulate ACTH and

corticosterone levels (Kim et al., 1999), inhibit the absorption of vitamins A and E in chicks and iron in the rat (Johnson et al., 1986; Southon et al., 1988a; Jenkins and Atwal, 1994). Interestingly they have been reported to increase mineral excretion of sodium, iron, magnesium and zinc (West, 1979; Freeland et al., 1985; Southon et al., 1988b), as well as change the circadian rhythm of feeding behavior (Petit et al., 1995).

On the other hand, other studies reported no adverse effect on growth after feeding fenugreek seeds containing steroid saponins (Ishaaya et al., 1969) and soybean triterpenoid saponins (Petit et al., 1995) at concentrations as much as five times the concentration in a normal soybean-supplemented diet of chicks, rats and mice. Yucca and quillaja saponins are classified as generally recognized as safe (GRAS) and are approved for food use in the USA (Fenwick et al., 1992).

In chapter V we reported that it was not clear which specific compounds in guar meal contributes to the growth inhibitory effects in the broiler chick diets. Most likely candidates for growth and feed efficiency inhibition are residual guar gum and triterpenoid saponins. In this study, the results obtained indicate that there are negative feed efficiency, body weight and growth rate effects associated with adding 0.125% saponin-rich guar meal extract to the diet of chicks.

Summary and Conclusions

The *Clostridium perfringens* challenge of chicks was unsuccessful in creating necrotic enteritis in this study. There were no differences between negative and positive control groups in all parameters measured. Adding 1% Guar gum in the diet of chicks

challenged with *Clostridium perfringens* significantly increased the colony forming unit numbers above the colony forming unit numbers of *Clostridium perfringens* in digesta of both negative and positive control treatment groups. Body weights at 21 d, and body weight gains during 14 to 21 d and 0 to 21 d in birds challenged and fed 2.5% guar meal or 0.125% saponin-rich guar meal extract were significantly lower than those of both negative and positive control groups during the same periods. Weekly feed conversion ratio of chicks challenged and fed 0.125% saponin-rich guar meal extract were higher than those of both negative and positive control treatment groups, but not different from the guar meal and saponin fed treatment groups during the 15 to 21 d period. Results suggest that saponins could contribute to the observed inhibion of growth in chicks fed guar meal.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Prophylactic use of antibiotics is no longer desirable due to increased concerns regarding antibiotic resistance. Recent research has focused on antibiotic alternatives to prevent and treat diseases such as use of probiotics, prebiotics and organic acids. Many pharmaceutical compounds used to protect and treat human and animal health originated from plant extracts. Among these plant compounds are saponins from quillaja and yucca that have several beneficial effects on animal health. Saponins are glycoside compounds distributed in many plants, lower marine animals and some bacteria. They consist of a fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) or steroid (C-27) attached with one or more sugar side chains (glycone) at different carbon sites of the aglycone. Saponins have many biological properties such as hemolytic, antibacterial and antiprotozoal activities.

Guar (*Gyamopsis tetragonoloba*) is a drought tolerant annual legume mainly grown for the guar gum (galactomannan polysaccharide) that has many industrial and food processing applications. Guar meal, a combination of hull and germ fractions as a by-product of guar gum manufacture contains 5-13% saponin by weight of dry matter, 13-18% residual galactomannan gum, and 33-47.5% crude protein that makes it potentially useful as an ingredient for poultry feed. Guar meal has characteristics of a useful feeding ingredient that could also have antimicrobial activity because of galactomannan and saponin content. Research has evaluated neither the hemolytic nor

antimicrobial activities of saponin-rich guar meal extracts. Therefore, the objectives of this research were to isolate saponin-rich extracts from guar meal to determine in vitro antibacterial activities against relevant gram-positive bacteria (*Staphylococcus aureus, Clostridium perfringens* and *Lactobacillus spp*.) and gram-negative bacteria (*Salmonella* Typhimurium and *Escherichia coli*) in a dose dependent manner compared with commercial quillaja, yucca and soybean saponins. Also, anticoccidial and anti-*Clostridium perfringens* activities were evaluated in vivo by adding guar meal, purified guar gum or saponin-rich extracts to feed of chicks challenged with the disease organisms.

Results showed that butanol extracts from guar meal yielded 4.8 ± 0.6% crude saponin by weight of the original material. Butanol extract purified by reverse-phase C-18 flash chromatography eluted 2 peak fractions with 20% methanol, and 1 peak each with 60, and 100% methanol to yield averages of 1.72 ± 0.47, 0.88 ± 0.16, 0.91 ± 0.16 and 1.55 ± 0.15% crude saponin by weight of the original material, respectively. Further normal-phase HPLC purification of the100% methanol saponin-rich guar meal extract eluted 4 peaks at 16, 39, 44 and 46 min. Hemolytic activity was observed in the 100 % methanol guar meal extract, sub-fractions of methanol extract eluted at16 min, and F and G guar fractions prepared independently by Dr. Mark Berhow. The 100% methanol extract was most effective against *Staphylococcus aureus, Escherichia coli, Lactobacillus spp*. and *Salmonella* Typhimurium at the concentrations tested. The 20 and 60% methanol extracts were neither hemolytic nor antibacterial, but stimulated *Lactobacillus spp*. at concentrations more than or equal to 0.39 mg/mL. The 100%

methanol guar meal extract and commercial quillaja saponins were significantly higher in both hemolytic and antibacterial activities against all bacteria tested than commercial yucca and soybean saponins. Soybean saponin had no antibacterial activity against any of the bacteria tested.

Adding 5% guar meal to diets of chicks challenged with *Eimeria tenella* reduced the infection severity with respect to oocysts shed per gram feces and bloody diarrhea. However, adding 5% guar meal in the diet decreased body weight, body weight gain and feed efficiency. The *Clostridium perfringens* challenge was unsuccessful in creating necrotic enteritis in this study. There were no differences in intestinal lesion scores, feed intakes or mortality rates among all dietary treatments. No differences were observed between negative and positive control groups in all parameters measured in this study. Adding 1% Guar gum in the diet of the chicks challenged with *Clostridium perfringens* significantly increased the colony forming unit numbers of intestinal contents. Adding either 2.5% guar meal or 0.125% saponin-rich guar meal extract to challenged chicks reduced body weights and body weight gain, but 0.125% saponin-rich guar meal extract increased feed conversion ratio. Results showed that guar saponin may be the main antinutritional compound in guar meal, however saponin-rich guar meal extract exhibited antimicrobial activity. Guar meal showed anticoccidial activity against *Emeria tenella* and saponin-rich guar meal extract exhibited antibacterial activity. Thus, guar products may have potential as an antibiotic alternative.

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Selected Publications

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