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The effect of Quillaja Saponin on the Growth and Performance of Nile Tilapia (*Oreochromis niloticus*)

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The effect of Quillaja Saponin on the Growth and Performance of Nile Tilapia (*Oreochromis niloticus*)

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Jerusalem-Palestine

Dedication

My deep thanks to my parents, who always encouraged me to aim high in life, and without whose teachings, sacrifice, patience and struggle, I would never have reached where I am today.

I'm deeply grateful to my other half, my wife, for her love and support that helped me to keep going, especially during those very often difficult times of frustrations.

I can't also forget my kids Mohammad, Adam and my lovely Hala.

Shaher Jawabra

Declaration

I certify that this thesis submitted for the degree of Master in Science is a result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: …….

Shaher Abed Al-Rahman Jawabra.

Date: 17/11/2011

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Abstract

The experiment was carried out in Al-Quds University aquaculture laboratory started on October 2010 until June 2011. About 750 fish nine days old were placed in the flow through system in that was designed to maintain optimum water quality by flushing out the remaining nutrients, nitrite and ammonium. The flow through system consist of 25 aquaria, each one has 30 female fry Tilapia fish nine days old when the experiment was started. The fish were divided into 5 groups A: control feeding., B: control feed plus 2 ppt saponin 10% for 5 weeks, C: control feed plus 2 ppt saponin 10% continuous feeding, D: control feed plus 2 ppt saponin 25% for 5 weeks, E: control feed plus 2 ppt saponin 25% continuous feeding. When the first 5 weeks ended, groups B and D were merged with control and stopped saponin feeding for them, but with keeping the isolation from the others. Weighting the fish was done weekly in order to calculate the feeding amount and also to notice the growth rate. The feeding amount depends on the fish weight. Results showed that, fish that were fed on feed with 25% saponin in the first five weeks (critical period) exhibited significantly higher body weight gain percentage (BWG)%, specific growth rate percentage (SGR) %, and lower feed conversion ratio FCR compared to control and fish fed with 10% saponin. On the other hand no significant differences were found in growth rate (GR) between different groups during continuous saponin feeding. Continuous saponin feeding did not affect FCR, GRs parameters, but average body weight, and BWG% were higher with 25% continuous saponin group, which could be contributed to saponins effect on intestinal digestion and absorption or attributed to other mechanisms that limit its availability to systemic circulation. There was no proportion of males and females in all groups. All the fish were female and they put eggs. In the current experiment the 25% saponin used is more refined and highly pure with greater biological activity than the 10% saponin. Thus it is clearly obvious that Quillaja 10% saponin sigma preparations neither served as aromatase inhibitors nor as growth enhancers. Higher doses of Quillaja 25% saponin sigma preparations could be required to achieve sex inversion or it did not show the potential to serve as aromatase inhibitors. Since its use at very high doses is very expensive and our aim in aquaculture is to increase nile tilapia productivity using natural plant products with low input costs, it is recommended to be used only as growth enhancers with no role in sex inversion.

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Abbreviations

Abbreviation	
BW	Body Weight
BWG	Body Weight Gain
CO	Cytochrome Oxidase
CP	Crud Protein
DF	Degree of freedom
DO	Dissolved Oxygen
FAO	Food and Agriculture Organization
FCE	Food Conversion Efficiency
FCR	Food Conversion Ratio
GH	Growth Hormone
GR	Growth Rate
IGF	Insuline –Like Growth Factor
LDH	Lactate Dehydrogenase
LH	Leutinizing Hormone
MT	Methyltestosterone
MW	Molecular Weight
QS	Quillaja Saponin
SGR	Specific Growth Rate
TDS	Total Dissolved Solids
TS	Trigonella Saponin

Unit

°C	Celsius
L	Liter
mg	Milligram
mg/Kg	Milligram per Kilogram
min	Minute
ppm	Part per million
ppt	Part per thousand
TDS	Total Dissolved Solids

Chapter One

1.1 Tilapia history and biology

The culture of Nile tilapia (*Oreochromis niloticus*) can be traced to ancient Egyptian times as depicted on bas-relief from an Egyptian tomb dating back over 4000 years, which showed the fish held in ornamental ponds (FAO). Worldwide harvest of farmed tilapia has now surpassed 800,000 metric tons, and tilapia are second only to carps as the most widely farmed freshwater fish in the world. The Nile tilapia (*O. niloticus*) was one of the first fish species cultured. Fast growth, tolerance to a wide range of environmental conditions (such as temperature, salinity, low dissolved oxygen, etc.), resistance to stress and disease, ability to reproduce in captivity and short generation time and feeding on low trophic levels and acceptance of artificial feeds immediately after yolk-sac absorption make them ideal for aquaculture, especially in developing countries (FAO).

Tilapia has become the most commercially cultured fish in the relatively short period since its introducing, and the most adaptable to environmental changes (Francis et al, 2001). Positive aquacultural characteristics of tilapia are their tolerance to poor water quality and their ability to eat a wide range of natural food organisms. Nile tilapia is a tropical species that prefers to live in shallow water. The preferred temperature ranges from 31 to 36 ℃. It is an omnivorous grazer that feeds on phytoplankton, aquatic plants, small invertebrates, benthic fauna and detritus.

There are four major species of Tilapia used in aquaculture. Species most often cultured are Nile Tilapia, Red Tilapia, Mozambique Tilapia and their hybrids. The species of the Genus Oreochromis are maternal mouth brooders and exhibit parental care. Under fast growing conditions they attain maturity in three months at a weight of 60 to 100 gm. Sex determination of tilapia is a very flexible process subjected to genetic, environmental (temperature), and behavioral and physiological factor (Devlin and Nagahama, 2002). One disadvantage of Tilapia culture is that when male and female produce a large amount of fry, this result in overproduction and competition for diet, so few of them will reach the marketable size. Commercial tilapia production generally requires the use of male monosex populations. Male tilapias grow approximately twice as fast as females. It is

therefore necessary to reverse the sex of female fry. This production relies on all male (or monosex) populations in order to avoid pond overcrowding due to their precocious sexual maturity and continuous reproduction, associated to an elaborated parental care and to benefit from male's higher growth rate (Baroiller and Jalabert, 1989). This is possible because tilapia do become sexually differentiated for several days after yolk sac absorption. If female tilapia receives a male sex hormone (17 α methyltestosterone, MT) in their feed, they will develop as phenotypic males. Several studies have demonstrated that steroid hormones can influence sex differentiation in non mammalian vertebrates and it has been hypothesized that male and female sex differentiation are driven by androgen and estrogen hormones, respectively. Estrogen biosynthesis is mediated by the steroidogenic enzyme cytochrome P450 aromatase, which converts androgens to estrogens. (Afonso et al, 2001). Though use of MT is effective and widespread, this practice may have undesired effects on human health and the environment. As a result of that, there is an immediate need for finding a more environmentally friendly substitute. An alternative solution may be the addition of saponins to the fish diet since these compound have been reported to have the ability to alter the plasma level of hormones that regulate reproductive activity (Tamura et al., 1997). Saponins are steroidal or triterpenoidal glycosides, common in a large number of plants and plant products that are important in both human and animal nutrition. Saponins have been found to significantly affect growth, feed intake and reproduction in animals (Golan et al, 2008).

1.2 Tilapia Environmental requirements

In order to enable fish farmers and farm managers to determine the chemical, biological and physical processes that take place in tilapia farms, and, to take the proper and correct supporting managerial decisions, It is necessary to understand the major water quality parameters and their interrelationships, which affect fish growth and health and determine the failure or success of overall culture practices. The major environmental factors affecting tilapia are temperature, salinity, dissolved oxygen, ammonia and nitrites, pH, photoperiod and water turbidity (Francis et al, 2001).

1.2.1 Temperature:

The physiology, growth, reproduction and metabolism of tilapia are affected by temperature. It is important in temperate and subtropical regions, which are characterized by seasonal fluctuations in water temperature. Tilapia are thermophilic fish and known to tolerate a wide range of water temperatures. for the normal development, reproduction and growth of tilapia, the temperature range is about 20 to 35°C, depending on fish species, with an optimum range of about 25–30°C (Balarin and Haller, 1982). However, a big difference in the growth and feed efficiency of tilapia may occur even in this narrow range of water temperatures. The effects of three water temperatures (24, 28 and 32°C) (lying within the optimum range of tilapia tolerance) on the growth and feed conversion of Nile tilapia fry, in a recirculating system was studied. The growth of the fish at 28°C was almost double the growth at 24 and 32°C (El-Sayed and Kawanna, 2006).

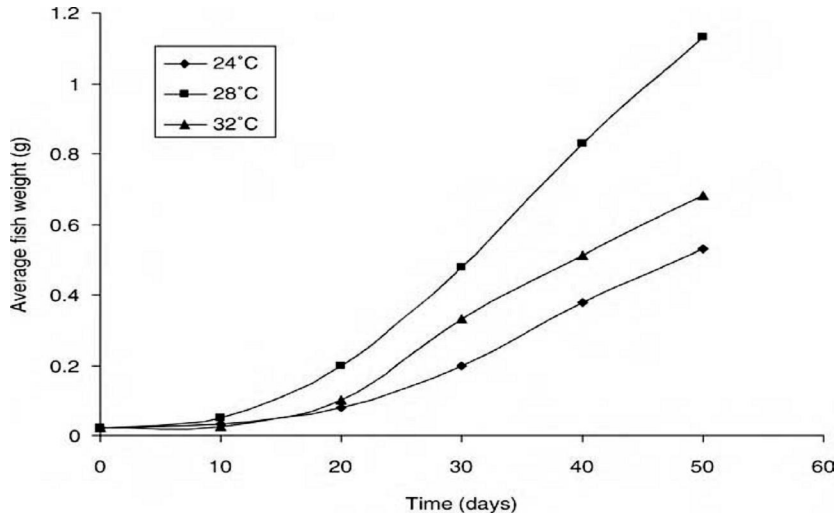


Fig. 1.1 :Effect of water temperature on weight gain of Nile tilapia fry reared in an indoor, closed system (A. El-Sayed and Kawanna).

Only for brief periods Tilapia can also tolerate temperature as low as 7–10°C, (Balarin and Haller, 1982). Long period of exposure to this low temperature can lead to mass mortality, below 20°C Tilapia feeding is sharply reduced and they stop feeding at about 16°C, at 12°C severe mortality occurs (Balarin and Haller, 1982). Tilapia can tolerate relatively high water temperatures for a long time. The upper lethal temperature limits for tilapia

vary from one species of tilapia to another, but it has been reported that most tilapias cannot tolerate water temperatures above 40–42°C (Balarin and Haller, 1982). It has been recognized that the further the geographical location from the equator, the more tolerant to cold are Nile tilapia (Sifa et al., 2002). A phenomenon related to natural selection process.

1.2.2 Salinity

Tilapia, despite being freshwater fish, they are believed to have evolved from marine ancestors (Kirk, 1972). This may explain the ability of most tilapia to tolerate a wide range of water salinity. The pressure to develop aquaculture in brackish water and seawater was increased as a result of competition for fresh water between agriculture and urban activities. The first proposal to think of for aquaculture in brackish water is tilapia. It can normally grow and reproduce in brackish water. Some species at very high water salinity can even grow and reproduce. salt tolerance depends on tilapia species(table 1.1), environmental factors, strains and size, adaptation time, and geographical location (Balarin and Haller, 1982).

Table 1.1: Salinity tolerance of some tilapia species (‰).

Species	Upper limit (direct transfer)	Upper limit (gradual transfer)	Optimum limit	Remarks	Reference
O. niloticus	**18** [1]	**36** [1]	**5–10** [2], **15** [3]	**Reproduce at 13.5–29‰** [4]	[1](Al-Amoudi, 1987a), [2](Payne and Collinson, 1983), [3](Alfredo and Hector,2002), [4](Balarin and Haller, 1982)
O.mossambicus	**27** [5]	**120** [6]	**17.5** [7]	**Spawn at up to 49‰** [8]	[5](Al-Amoudi, 1987b), [6](Whitefield and Blaber, 1979), [7](Canagaratnam, 1966), [8](Popper and Lichatowich,1975)
O. aureus	**27** [5]	**54** [4]	**10–15** [9]	**Reproduce at 5-20‰,**	[9](Perry and Avault, 1972), [10]McGeachin et al., 1987)

The reproduction of tilapia and gonadal development were also affected by water salinity. and spawning of Nile tilapia occurred at salinities of 17–29‰, while the starting of reproduction was delayed with increasing water salinity from 25 to 50‰, and reproduction stopped completely at salinity above 30‰.

1.2.3 Dissolved oxygen

One of the environmental factors affecting feeding, growth and fish metabolism is dissolved oxygen (DO). DO fluctuation is affected by photosynthesis, respiration and temperature. These factors must be fully considered where DO is concerned. Good DO range produces the best fish performance, other metabolic activities of fish like respiration and growth could be limited while low DO levels (Tsadik and Kutty, 1987). Tilapia are known to tolerate with a very low levels of DO. Most tilapias can tolerate DO levels between 0.1–0.5 mg/l for varying periods of time (Tsadik and Kutty, 1987). Also they can survive even with 0 mg/l by going to the surface air, they can suffer and will have high mortality if they could not allowed to reach the surface. On the other hand, it can tolerate with supersaturation (up to 400%) when there is a high concentration of oxygen as a result of high photosynthesis of phytoplankton and algae. Rate of DO in the water is affected by water temperature. Thus, increasing water temperature reduces DO, which leads to increased respiration rate and oxygen consumption in tilapia, because under increased water temperature the rate of metabolism increased. The rate of oxygen consumption in tilapia increased from 0.74 to 0.97 mg/l/h with increasing water temperature from 37 to 42°C (Franklin et al.,1995). at oxygen saturation of 25–32% (at temperature ranging from 15 to 30°C)respiration of tilapia was independent of DO, while the metabolic rate became dependent on oxygen availability below these saturation levels, and mortality occurred when DO remained below 20% saturation for more than 2–3 days. Oxygen consumption decreased when carbon dioxide increased in the water. However, tilapia can also withstand very high levels of CO_2, ranging from 50 to 72.6 ppm.

1.2.4 Nitrite

When Ammonia is oxidized, it produces nitrite (NO_2^{2-}) and then converted into nitrate (NO_3^{2-}) through bacteria grow on suspended organic matter. The bacteria remove the organic matter by feeding on it. Long exposure to Nitrate(NO_3^{2-}) in a high concentration

may decrease immune response and induce mortality in tilapia, but in general it is relatively nontoxic (Plumb, 1997). On the other hand Nitrite (NO_2^{2-}) is highly toxic to fish, including tilapia, because it can affect the physiological functions of the fish. Tilapia tolerance to nitrite is influenced by fish size. (Atwood *et al.*, 2001) found that small-sized Nile tilapia (4.4 g) were more tolerant to nitrite than large fish (90.7 g). In order to protected both small and large fish from nitrite toxicity. The addition of a chloride source ($CaCl_2$ or NaCl) to culture water can save the fish.

1.2.5 Ammonia

Nitrogenous wastes of fish are excreted in the form of ammonia. Excreted ammonia exists in un-ionized NH_3 and ionized form NH_4^+, which is nontoxic. There is a relationship between the toxicity of ammonia, DO, CO_2 and pH. With decreasing DO the toxicity increases and decreases with increasing CO_2. Also the ammonia toxicity depends on fish size, and fish species. when Nile tilapia exposed to ammonia, this will result in low number of red blood cells and have hemolytic anemia, leading to a significant reduction in blood oxygen content.(Ahmed *et al*., 1992). The toxic concentration of ammonia and its negative effect on the growth performance ranges from 0.07 to 0.14 mg/l. In addition to ammonia concentration, the effect of ammonia on tilapia is related to water pH and exposure period. The limit of water quality parameter was summarized in table 1.2.

Table1.2 : Limits and optima of water quality parameters for tilapia

Parameter	Range	Optimum for growth	Reference
Salinity, parts per thousand	Up to 36	Up to 19	(El-Sayed, 2006)
Dissolved oxygen, mg/L	Down to 0.1	> 3	(Magid and Babiker, 1975); Ross, 2000)
Temperature, °C	8–42	22–29	(Sarig, 1969; Morgan 1972; Mires,1995)
pH	3.7–11	7–9	(Ross, 2000)
Ammonia, mg/L	Up to 7.1	< 0.05	(El-Shafey, 1998; Redner and Stickney, 1979)

1.2.6 pH

Nile tilapia can survive at a range of pH between 4–11 (Wangead *et al*., 1988), but they die at pH 3.5 and 12. both fingerlings and adults died at pH 2–3 within 1–3 days. Both size groups tolerated pH 4–5 very successfully, and had survival and growth rates similar to the control group (pH 7) after 60–70 days. However, adult fish were more resistant to low pH, with a survival rate of 86.6, 100 and 100% at pH 4, 5 and 7, respectively, whereas the survival of fingerlings was 57.8, 82.2 and 84.5%, respectively, at the same pH values. (Wangead *et al*., 1988).

Low or high water pH may lead to behavioral changes, damage of gill epithelial cells, reduction in the efficiency of nitrogenous excretion and increased mortality. Fingerling and adult Nile tilapia exposed to pH 2–3 within 1–3 days showed rapid swimming, lack of body position and opercular movements, surfacing of air, and mass mortality. (Wangead et al., 1988). In acidic water (pH 4), standard metabolic rate, maximum metabolic rate and oxygen consumption in tilapia decreased.

1.2.7 Photoperiod

Photoperiod can affects many physiological functions such as daily activity, promoting fish growth and metabolic rates, body pigmentation, sexual maturation and reproduction, depend on fish species and size (El-Sayed and Kawanna, 2006). The response of Nile tilapia to photoperiod cycles depends on fish developmental stage and sex (El-Sayed and Kawanna, 2006).

Larval stage was more sensitive to photoperiod than fingerling and juvenile stages. Fish fry subjected to long photoperiods (24 and 18 h) had significantly better performance than those exposed to intermediate or short photoperiods (12 or 6 h).

Few studies have considered the effects of light intensity and photoperiod on reproductive, indicated that low light intensity leads to low spawning activity (Ridha and Cruz, 2000). Other study recommended for optimum seed production of Nile tilapia, the light intensity have to be 2500 lux and a photoperiod of 18 h/day. (Ridha and Cruz, 2000) with increasing photoperiod Gonad development, fecundity and spawning frequencies also tend to improve. Under normal day length (12 h light : 12 h dark cycles) the best reproductive performance is achieved.

1.2.8 Water Turbidity

Water turbidity originates from a number of sources including turbid source water; rainwater runoff containing clay materials; and the water and fish movements that cause a suspension of bottom mud. The high concentrations of suspended colloidal particles causes a reduction in fertilizer effect, water acidity and inhibit light penetration, and in turn adversely affect primary productivity in fish ponds with increasing water turbidity, fish growth, feed efficiency and survival were all significantly reduced, but the differences at the higher levels of 100–200 mg/l were not significant. Clay turbidity levels in earthen ponds should be kept below 100 mg/l. (Ardjosoediro and Ramnarine, 2002).

Chapter Two

2.1 Nutritional requirements

The low trophic level and the omnivorous food habits of tilapia make them a relatively inexpensive fish to feed, unlike other finfish, such as salmon, which rely on high protein and lipid diets based on more expensive protein sources like fish meal. In addition, they can tolerate higher dietary fiber and carbohydrate concentrations than most other cultured fish. To ensure high yield and fast growth at least cost, a well-balanced prepared feed is essential to successful tilapia culture. Slight variations exist among tilapia species, but nutrient requirements are primarily affected by the size of the fish (El-Sayed, 2006).

2.1.1 Lipids

Dietary lipids provide a major source of energy, facilitate the absorption of fat soluble vitamins, play an important role in membrane structure and function, serve as precursors for steroid hormones and prostaglandins, and serve as metabolizable sources of essential fatty acids. found that for tilapia up to 2.5 g, the optimum dietary lipid concentration was 5.2%, decreasing to 4.4% for fish up to 7.5 g. Jauncey (2000) suggested that to maximize protein utilization, dietary fat concentration should be between 8 and 12% for tilapia up to 25 g, and 6 to 8% for larger fish.

2.1.2 Vitamins and Minerals

Vitamins and minerals are essential for normal fish metabolism. Vitamin and mineral supplementation in the form of premixes may be beneficial in intensive systems, although most of these requirements are usually met naturally in extensive and semi-intensive pond cultures. However, specific requirements are not exactly known for all vitamins. Because of the limited knowledge and the uncertainty regarding vitamin requirements, it is difficult to make general recommendations as to what the optimal concentrations should be, but general minimum levels are commonly applied to feeds.

2.1.3 Protein

Fish do not have a specific requirement for crude protein (CP) per se, but rather they need a combination of essential amino acids. Therefore, the profile of dietary protein is important when formulating diets for tilapia. Dietary proteins are used continuously by fish for maintenance, growth, and reproduction functions. (Twibell and Brown, 1998). As with other warm water fish, tilapia require 10 essential amino acids that need to be supplied by the diet (table 2.1).

Table 2.1: Amino acid requirements (dry basis) of tilapia

Amino acid	**% of dietary protein**	
	O. niloticus	***O. mossambicus***
Arginine	4.20	2.82
Histidine	1.72	1.05
Leucine	3.39	3.40
Lysine	5.12	3.78
Valine	2.80	2.20
Threonine	3.75	2.93
Tryptophan	1.00	0.43
Phenylalanin	3.75	2.50
Isoleucine	3.11	2.01
Methionine	2.68	0.99

2.1.4 Carbohydrates

Fish do not have a specific requirement for carbohydrates, because amino acid and fatty acid precursors can supply the required glucose via gluconeogenesis. This does not imply that carbohydrates should not be included in tilapia diets, however carbohydrates provide a relatively inexpensive source of energy compared to protein, and their inclusion can improve the quality of pelleted feeds. Tilapia can effectively utilize carbohydrate levels up to 30 to 40% in the diet, which is considerably more than most cultured fish. (Shiau and Huang, 1990).

2.2 Tilapia Monosex Production

Mixed-sex culture of tilapia has been a common practice. During the past two decades intensive attention has been given to monsex culture of tilapia. High growth rates and feed utilization efficiency, high tolerance to environmental conditions, including temperature, salinity, low dissolved oxygen, etc, higher energy conservation, greater uniformity of size at harvest, better meal quality and appearance, and resistance to stress and diseases make them an excellent candidate for aquaculture.

The following methods have been used for the production of monosex tilapia:

2.2.1 Manual sexing

The technique of manual sorting is based on assessing the number of openings in the urinogenital papillae: the male has a single urinogenital opening, while the female has two separate openings. It was widely used in the past (Shell, 1967). It is an easy technique, but it is extremely laborious and stressful for the fish and often leads to inaccurate results due to the presence of females as a result of human error (Penman and McAndrew, 2000). Therefore, this method is rarely used and accuracy of this method ranges from 80 to 90% (Penman and McAndrew, 2000). Accuracy also increases with increasing fish size.

2.2.2 **Hormonal sex reversal**

2.2.2.1 **Oral administration**

Hormonal sex reversal has been widely used for producing monosex fish and sex determination and for aquaculture purposes. Steroid hormones or hormone analogues as well as non-steroid compounds are commonly used for producing monosex tilapia (table 2.2).

Table 2.2: Steroid hormones, hormone analogues and non-steroid compounds used for producing monosex tilapia.

Hormone (compound)	Abbreviation
17α-methyldihydrotestosterone	MDHT
17 α -methyltestosterone	MT
17 α -ethynyltestosterone	ET
17 α -methyl-5-androsten-3-17β-diol	-
Androstenedione	AN
17 α -ethymyloestradiol	EE
Oestradiol-17 β	E2
Fadrozole	F
Trenbolone acetate	TBA
11 β -hydroxyandrostenedione	11 β -OHA4
Aromatase inhibitor	AI
Diethylstilboestrol	DES
Tamoxifen	-
Acriflavine	-
Mibolerone	MI

at very early larval stages. The hormones are generally incorporated into larval feeds and administered to undifferentiated larvae for sufficient time to enable sex reversal. The use of hormones has been under increasing public criticism due to their possible health and environmental impacts. As a result, the use of hormones for sex reversal of tilapia is either licensed (in USA) or banned (in Europe) (Penman and McAndrew, 2000).

The most common and successful hormone used for tilapia sex reversal is 17α-methyltestosterone (MT) hormone (Penman and McAndrew, 2000;. The best results have

been reported at a dose of 30–60 mg/kg administered for about 25–30 days (Green *et al.,* 1997).

2.2.2.2 Immersion Techniques

When tilapia is treated with Oral administration of hormones for sex reversal, hormone traces from uneaten food and metabolites are often a major environmental concern. A successful alternative tool to overcome this problem is Immersing fish fry in hormone solution for short periods of time.

This method can decrease the treatment period and reduce the possible effects of the hormones on the workers (Gale *et al*., 1999). In spite of this, the use of the immersion technique for tilapia sex inversion has not been developed for practical and commercial use. The percentage of sex-reversed males produced by the immersion technique ranges from 60% to 100%, depending on fish species, type and dose of hormone and immersion period. The exposure of fish larvae to ultrasound can increase the transport of hormone from the water into the fish body, leading to a higher masculinization rate (Bart, 2002). Food safety and environmental issues should be considered when steroids are used in sex reversal of tilapia. The producer must have the obligation to be insured that it must have minimal negative effects on the environment. The use of drugs in the US in aquaculture is regulated by the Food and Drug Administration (FDA). Only a few drugs have been approved by FDA for use in aquaculture.

2.2.3 Genetic control

The best way to obtain all-male populations is through genetic control (Baroiller et al., 1999). Based on the first data on tilapia sex determination and differentiation, it has been possible to produce genetically “all-male populations” through the development of YY “supermales” (Baroiller et al., 1999). Nevertheless, this approach is unreliable and hampered by the very long procedure of producing and identifying putative YY male individuals. Moreover, sex determination has been shown to be more complex than a simple agents XX/XY mono factorial system.

2.2.4 Environment

Environmental factors (temperature, pH, density and social interactions) could influence the sex ratio in gonochoristic species. In fish, the main environmental factor influencing sex seems to be temperature. In tilapia, as thermosensitive species, male to female ratio increases when temperatures were higher than 32^0C (Baroiller and D'Cotta, 2001). Environmental factors such as temperature can impact growth and in turn the treatment duration needed. Temperature alone can also skew sex ratios. Water quality is a consideration when sex reversing tilapia. Most sex reversal is done in freshwater over a range of alkalinities and hardnesses. The literature does not suggest that efficacy is affected by alkalinity or hardness. Several species of tilapia such as *O. mossambicus* and *O. spilurus* can reproduce in brackish or full strength seawater and the fry can be sex reversed under such conditions. Water quality conditions that will allow good growth and survival of fry are appropriate for sex reversal also. Dissolved oxygen (DO) concentrations should remain above 4 mg/l to insure a strong feeding response. The fry will tolerate lower levels but are stressed and more susceptible to diseases.

2.2.5 Non-Hormonal Sex reversal

A serious drawback for tilapia farmers who intend to sell their products internationally is that administering hormones to food fish or import of hormone treated fish is, however, forbidden in several countries (among them the European Union) for concerns regarding consumer health and safety of the aquatic environment. Endocrine active plant derived substances, showing a similar effect on tilapia or other food fish as MT does, would be an alternative to synthetic hormones and would have a greater acceptance among consumers and will most likely possess a lower environmental risk. For that reason the interest in plant derived alternatives to natural or synthetic hormones is internationally increasing. One candidate group of plant secondary compounds are saponins which are glycosides produced by many plant families (Fenwick et al. 1991) and in some marine invertebrates. They consist of an either triterpenoidal or steroidal aglycone (sapogenin) and a highly variable sugar moiety resulting in a great variety of saponins. In general, triterpenoidal saponins are predominant in cultivated crops while steroidal saponins occur mostly in wild plants used as herbs or for medicine (Fenwick et al. 1991).

Chapter Three

Saponins

3.1 Saponins structure

Saponins are the major group of glycosides that are highly distributed in higher plants, consist of a polycyclic aglycones attached to one or more sugar side chains. The aglycone part, which is also called sapogenin, is either steroid (C27) or a triterpene (C30). Figure 3.1.

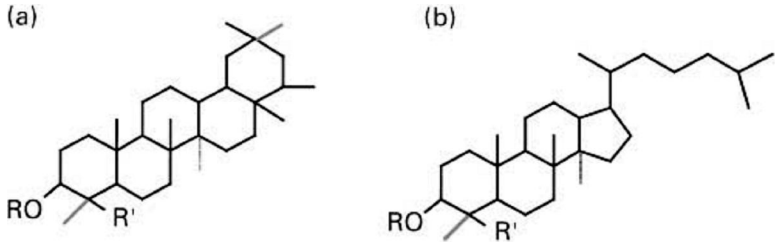


Figure 3.1: basic structure of sapogenin: (a) a triterpene and steroid(b).

They are naturally occurring surface-active glycosides mainly produced by plants, but also by lower marine animals and some bacteria (Yoshiki et al. 1998). They derive their name from their ability to form stable, soap-like foams in aqueous solutions. This easily observable character has attracted human interest from ancient times. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid in nature. The aglycone may contain one or more unsaturated C–C bonds. The oligosaccharide chain is normally attached at the C3 position (monodesmosidic), but many saponins have an additional sugar moiety at the C26 or C28 position (bidesmosidic). The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone. Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have provoked considerable clinical interest in these substances. The foaming ability of saponins is caused by the combination of a hydrophobic (fat-soluble) sapogenin and a hydrophilic (water-soluble) sugar part. A wide

type of plants contain saponins in leaves, stem, bark, fruit and root. Also they are found in a wide variety of food including asparagus, bean, blackberries, peas, potatoes and tea (Dini et al., 2001a). The basic function of them is unknown, but they have been shown to protect plant from insects attack and prevent mould (Barr et al., 1998). Saponins have many positive effects such as, they are the major active components in many traditional herbal medicine, antiviral activity, cholesterol lowering activity, immunostimulant and anti diabetic activity (Petit et al., 1993). They are believed to form the medical properties of many plant drugs (Estrada et al., 2000).

3.2 Occurrence

Saponins occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties (Fenwick et al. 1991). Triterpenoid saponins have been detected in many legumes such as soyabeans, beans, peas, lucerne, etc. and also in alliums, tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenugreek, yucca and ginseng. One example of an extensively studied group of triterpenoid saponins is produced from Quillaja saponaria, a tree native to the Andes region. The bark was peeled off and extracted with water by the indigenous peoples as a shampooing agent, and by the Shamans as an overall curing agent.

3.3 Effects on cell membranes

Permeabilisation and effects on other membrane properties. A large number of the biological effects of saponins have been ascribed to their action on membranes. In fact, their specific ability to form pores in membranes has contributed to their common use in physiological research (Choi et al. 2001). Saponins have long been known to have a lytic action on erythrocyte membranes and this property has been used for their detection. The haemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol with which they form insoluble complexes (Bangham & Horne, 1962). The amount of glycosides required for

permeabilisation is much lower for cholesterol-rich lipid layers than cholesterol-free membranes (Go¨gelein & Hu¨by, 1984).

3.4 Effects on animal reproduction

The negative effects of saponins on animal reproduction have long been known and have been ascribed to their abortifacient, antizygotic and anti-implantation properties. Commercial pharmaceutical grade saponins caused abortion or death or both in rabbits, goats and cows when administered intravenously at concentrations above 2·3 mg/kg body weight. Saponins were found to be extremely strong stimulators of luteinising hormone release from cultured hypophysial cells (El Izzi et al. 1989; Benie et al. 1990) but their action was neutralised in the presence of serum indicating a passive membrane-permeabilising effect in this case.

Chapter Four

Literature Review

(Francis et al, 2001) studied the effects of *Quillaja* saponins on growth, metabolism, egg production and muscle cholesterol in individually reared Nile tilapia. Results showed that The average body mass gain during the 14-week experimental period of the Saponin 300 mg/kg group was 245% over the initial body mass, significantly(P<0.05) higher than that of the control group(188%), but not that of the S150 mg/kg group (226%). There was a 26% difference between the final average body mass of the C and S300 mg/kg groups and a 16% difference between the C and S150 mg/kg groups. The average metabolic growth rates was the highest in the S300 group at the end of the experiment, and the lowest in the C group. The growth rate, however, did not follow a uniform pattern during the experimental period. The highest average metabolic growth rates was in the S150 mg/kg group(16.1).after 3 weeks of feeding the experimental feeds, and lowest in the C group (13.6); P value of the difference between the two was exactly 0.05, with the figure for the S300 mg/kg group lying in between (14.7). The three female fish in the control group and one out of the two female fish in the S150 group regularly spawned at predictable time intervals. The remaining female fish, i.e. one out of two of the S150 group and both fish of the S300 group, never spawned during the entire 14-week experimental period. The ovaries of the non spawning fish were full of apparently normal eggs when the fish were dissected at the end of the experiment. The quantity and morphology of the eggs produced by the S150 fish were similar to those of the control.

Another experiment studied the effect of Quillaja saponins as a natural growth promoter for fish (Francis et al., 2005). Results showed that food containing QS (150 and 300 mg kg−1 diet) had significantly higher rate of body mass gain (Francis et al., 2001b, 2002a). The average final body mass of carp fed QS was about 18% higher and that of tilapia and was more than 20% higher than that of fish that had similar average weights at the start of the respective experiments but which did not receive QS. The growth-promoting effects of QS was most pronounced at the level of inclusion of 150 mg kg−1 in diets for carp, whereas the dietary level of 300 mg kg−1 induced maximum effects in tilapia. The absolute increase in weight in Nile tilapia was greater compared to the control even at

higher dietary levels of 700 mg kg−1 (Francis et al., 2002c).Sexually mature female tilapia consuming a diet containing 300 mg QS kg−1 did not spawn over a period of more than 3 months, whereas fish fed the control diet and reared under similar conditions spawned regularly. This observation was followed up by conducting laboratory experiments and field studies to explore further the effects of dietary QS on tilapia reproduction. Regularly spawning adult tilapia when put on a diet containing 300 mg QS kg−1 stopped egg laying from the next ovulation cycle onwards (G. Francis and K. Becker, 2003). In another experiment, the sex ratio of tilapia larvae fed continuously over a 6-month experimental period a diet containing 700 mg QS kg−1 deviated significantly from the normal 50:50 ratio in favour of males (Francis et al., 2002c). This deviation from the normal sex ratio in favour of males was also evident in the treatment groups receiving lower quantities of QS (150 mg kg−1).

4.1 Hypothesis

Intensive attention has been given to monsex (male) culture, since their higher growth rates and uniformity of size with better feed utilization efficiency and energy conservation, make them an excellent candidate for aquaculture. The most common method for producing monsex (male) culture is hormonal sex reversal with methyl-testosterone (MT). Since the application of synthetic hormones in animal feed is prohibited in many countries including the whole of the EU, due to environmental and health concerns, endocrine active plant derived substances with a similar effect could serve as a more environmentally friendly substitute for MT. In vitro studies showed that saponins inhibit the aromatase activity, the rate limiting enzyme that converts androgens to estrogens needed for ovarian differentiation in Nile tilapia. In previous experiments saponin supplementation in fish diets has shown a potential to influence growth and reproduction success. Subsequent field investigations in Bangladesh were unable to confirm this, but fish from the experiment that were left to grow to commercial size did not appear to reproduce. The present study tested the effects of quillaja saponins on growth and fertility of Tilapia as continuous feeding supplement.

Our hypothesis is " *Quillaja* saponins (QS) when fed to Nile Tilapia can improve growth performance, and are capable of causing sex inversion in larvae and/or suppression of reproduction in adults.

4.2 Objectives:

1- To enhance the growth performance by natural, fully biodegradable feed additives.

2- To control high reproduction by environmentally friendly and not legally restricted means (to replace synthetic testosterones).

Chapter Five

Materials and methods

5.1. Experimental system and animals

The study was carried out during the first five weeks in Al-Quds University aquaculture research laboratory using the flow through system. Then the fish were transferred to the laboratory ponds with approximate salinity of 4 TDS (total dissolved solids) in Jericho city. The experiment was started in October 2010 and finished in July 2011, the fish were transferred Each of the original groups were moved into a separate "hapa" net. The flow through system was designed to maintain optimum water quality by flushing out the remaining nutrients, nitrite and ammonium. The flow through system consist of 25 aquaria, each one has 30 genetically female (XX) fry Tilapia fish nine days old when the experiment was started. The flow throw system thermostat is set to maintain temperature between 26 – 28 °C. The flow rate in the first 2 weeks was between 40 – 50 ml/min, increasing this amount with time. The water was aerated by putting an airstone in every box also in the main tank.

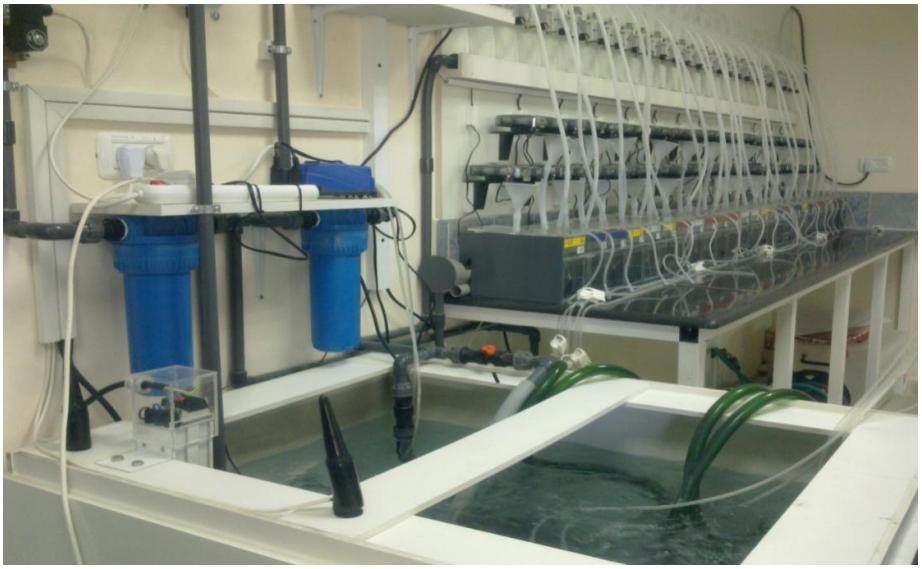


Figure 5.1 : The Flow throw System.

Ammonium and nitrite were checked twice daily by taking three samples randomly from three different aquaria, also the chlorine was checked in the main tank. After the 5th week, fish were transferred to Jericho ponds. Each group was separated in different pond. Before putting the experimental fish in ponds, other fish were placed in the same water type to insure that the well water is suitable for reproduction and for fish life.

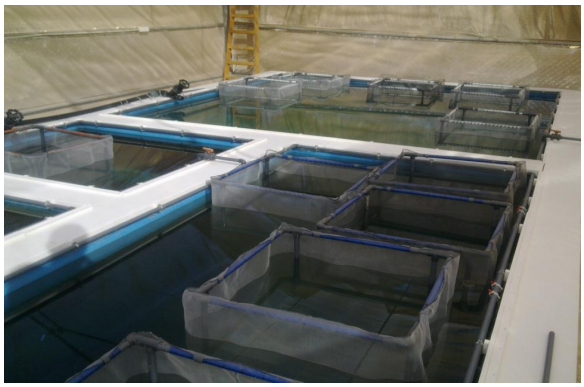


Fig. 5.2: Completely Different separation of " hapa nets"

5.2. The flow through system

In recirculating systems, saponin may remains in the water column long enough to influence sex ratios and growth rate. Water with saponin treated diet, and other with the same system with saponin free diet may be mixed and skewed sex ratios and growth rate. In order to avoid this interfering the flow through system was designed. The Main purpose of this system that water is passed through the system only once and then discharged to the sewage. The flow of water through the culture system supplies oxygen to the fish. Water quality within the culture system is maintained by flushing of contaminants and by replacing all system water before dissolved oxygen concentrations drop below minimum acceptable limits or contaminate concentrations (i.e. ammonia, solids, and carbon dioxide) can accumulate to above maximum acceptable limits.

The flow through system has many constituents such as: the 25 equal volume boxes, flow meters, automatic feeders, main big tank, biofilter, non biofilters, thermostat, digital thermometer, water pump, automatic water stopper…etc. The 25 aquaria were allocated in a random way.

5.3 Diet formulation and preparation

Commercial feed was used as the control diet. Saponin 10% (10% sapogenin, the aglycone part which is saponin compound without sugar chain) and saponin 25% (also 25% sapogenin) were purchased from Sigma corporation (Sigma-Aldrich is a leading Life Science and High Technology Materials company focused on enabling science to improve the quality of life. 3050 Spruce St. Saint Louis, Missouri, USA, Tel 800-325-5832, www.sigma-aldrich.com).

The experiment consisted of five treatment groups as follows:

A: control Diet.

B: control feed plus 2 ppt saponin 10% for 5 weeks.

C: control feed plus 2 ppt saponin 10% continuous feeding.

D: control feed plus 2 ppt saponin 25% for 5 weeks.

E: control feed plus 2 ppt saponin 25% continuous feeding.

The saponin 10% and saponin 25% are commercial preparations from the sigma corporation made from the park of Quillaja saponaria tree. In order to calculate the feed intake weighing the fish was done weekly to notice the growth rate also. The feeding amount was calculated as the table below depends on the fish weight.

Table 5.1 : most likely weights of fish during the first 4 weeks and recommended feeding level.

Week	Fish weight(mg)	Feeding % of biomass	Mg food /fish/day
1	10	30	3
2	40	20	8
3	100	15	15
4	250	10	25

After taking new weight results, food weighting process was done daily.

5.4 Water quality

During the experiment period, the water temperture ranged between 28-29 ºC, pH 8.4, total alkalinity 238.5mg/l, total hardness 291.2mg/l, soluble reactive phosphorous 109 mg/l, total nitrogen ammonia 2.68 mg/l, Nitrate 72mg/l, total phosphorous 35mg/l, total nitrogen 2.21mg/l, air stones were put in all aquaria inorder to keep dissolver oxygen >5 mg/l. Nitrite in all aquaria was kept in between 0.05 – 0.1 mg/l, ammonium <0.1 mg/l.

5.5 Growth performance

Growth performance and diet nutrient utilization were analyzed in term of percent body weight gain (BWG), growth rate (GR), feed conversion ratio (FCR), feed conversion efficiency (FGE), specific growth rate (SGR). The following formulas were used:

BWG (%) = 100* (final body weight –initial body weight) ⁄ initial body weight.

FCR = the weight of the feed fed to the fish along the study period ⁄ live body weight gain.

FCE = fresh body mass gain⁄the weight of the feed fed to the fish along the study period.

SGR (%) = 100* [ln (final body weight)-ln(initial body weight)] ⁄ no. of days

5.6 Statistical analysis

Data comparisons between the feeding groups were subjected to ANOVA using SPSS Program. The significance of observed differences was tested at $p < 0.05$.

Table 5.2: Numbers, Mean scores and standard deviation for the differences in using different saponin purification concentration during continuous diet period.

Saponin	N	Mean	Standard deviation
25%	29	20.10	9.03
10%	29	18.34	5.40
Control	29	17.52	4.67
Total	87	18.94	6.17

Table 5.3: One way analysis of variance for the differences in using different saponin purification concentration during continuous diet period.

Source	DF	Sum of squares	Mean square	F-value	Sig.
Between groups	2	188.25	94.128	0.135	0.874
Within groups	84	58734.22	699.217		
Total	86	58922.47	…….		

Data indicates that there are no statistical significant differences at $p \leq 0.05$ in the growth rate of fish during the continuous diet period.

Table 5.4: Numbers, Mean scores and standard deviation for the differences in using different saponin purification concentration during the first five weeks diet.

Saponin	N	Mean	Standard deviation
25%	5	0.11	0.08
10%	5	0.10	0.06
Control	5	0.11	0.07
Total	15	0.10	0.06

Table 5.5: One way analysis of variance for the differences in using different saponin purification concentration during the first five weeks diet.

Source	DF	Sum of squares	Mean square	F-value	Sig.
Between groups	2	0.001	0.000	0.038	0.043
Within groups	12	0.064	0.005		
Total	14	0.065	…….		

The above data indicates that there are statistical significant differences at $p \leq 0.05$ in the growth rate of fish during the first five weeks. The differences were in favor to the 25% group.

Chapter Six

Results and discussion

6.1 Average weight and growth rate under different experimental diets

Fish in all groups fed actively on the experemental diets and consumed the whole feed immediately. All the diets were equally acceptable, they did not show any abnormal behavior during the experimental period. There was no rejection of feed except in the later part of experemnt when the fish intestines were infected by nematodes, they stoped eating. Mortality was observed in the beginning as a result of high Chlorine in municipality water as we mentioned. After that few normal mortality was observed.

Table 6.1: growth performance and feed utilization of fish.

Group	Period (day)	Food weight consumed (g)	Initial body weight (g)	Final body weight (g)	Feed conversion ratio(FCR)	Specific growth rate SGR %	Percent body weight gain (BWG) %
Control (group A) first 5 weeks	35	0.554	0.01	0.218	2.54	8.80	2082
10% (group B)	35	0.489	0.01	0.197	2.48	8.50	1870
25% (group D)	35	0.534	0.01	0.241	2.22	9.00	2307
10% (continious) group C	280	153.4	0.01	86.45	1.77	3.23	864400
25% (continious) group E	280	175.3	0.01	98.36	1.78	3.28	983409
Control (continious)	280	155.1	0.01	89.83	1.73	3.25	898200

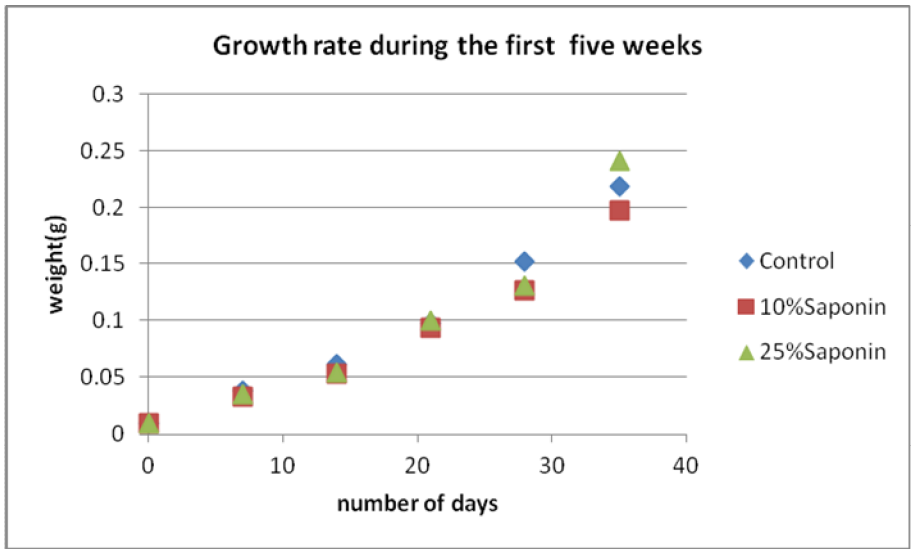
Feed conversion ratio (FCR) has a very important role in determination the efficiency of diet to fish. they measure the animal's efficiency in converting feed mass into increased

body mass. Also they are important calculations for the grower. They can be used to determine if feed is being used as efficiency as possible (Steven & Louis, 2002).

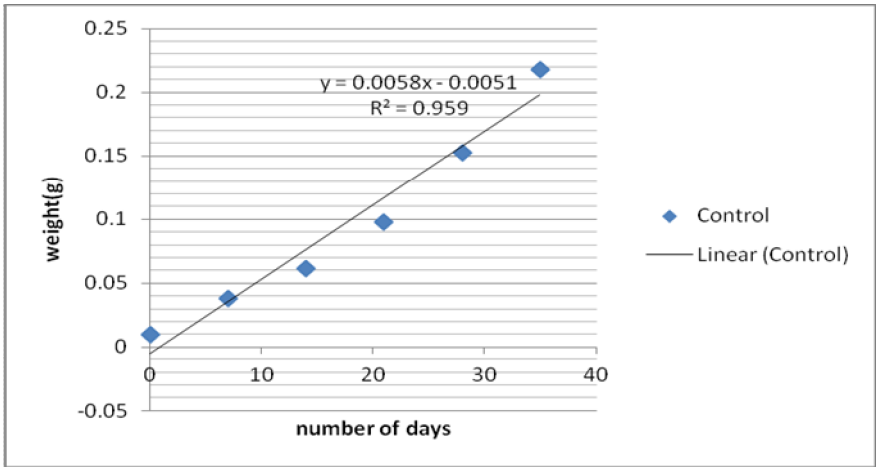
The efficiency of FCR related to better digestibility. The faster the growth the better the conversion efficiency. Fish with the highest biomass deposition rates that achieve their potential growth rates will produce much better food conversion efficiencies throughout their life than underachievers with the same potential. The growth and feed conversion ratio (FCR) of a fish is remarkable tool to compute the acceptability of feed. Fish that were fed on feed with 25% saponin in the first five weeks exhibited significantly ($p < 0.05$) higher body weight gain percentage (BWG)% (2307), specific growth rate percentage SGR% (9), and lower feed conversion ratio FCR (2.22) compared to control and fish fed with 10% saponin Table (6.1). On the other hand, fish fed with continuous 25% saponin over long period (280 days) gave higher percent body weight gain (BWG)% (983409), but feed conversion ratio FCR and were comparable to control and fish fed with 10% continuous saponin.

Fish treated with 25% Saponin during the first five weeks had higher growth rate GR compared to control. Figs. (6:1, 6:2, 6:3), table (6.4). Fish fed with 10% Saponin showed lower growth rate GR compared to control and 25% Saponin group figs (6:1, 6:2, 6:3, 6:4), table (6.4). Thus, feeding 10% Saponin did not affect growth and optimum dose for growth enhancement is to feed fish starting from 25% Saponin.

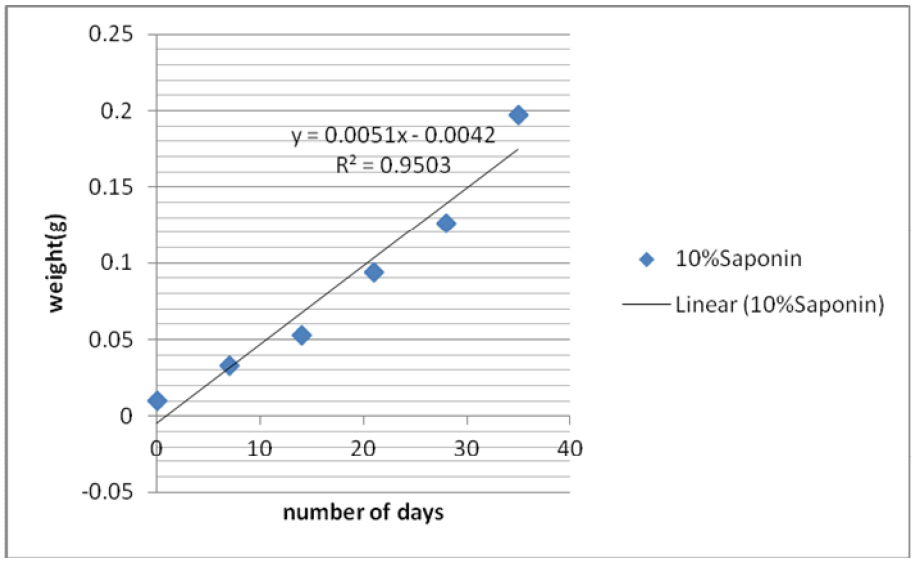
No significant differences were found in growth rate (GRs) between different groups during continuous saponin feeding, ($p > 0.05$) figs (6:5, 6:6, 6:7, 6:8, 6:9, 6:10, 6:11, 6:12), table (6.4), but the average final body weight of fish in continuous 25% saponin group tended to be higher than controls, although not statically significant ($p > 0.05$) Table 6.2. Among the treatment groups, the fish that received the 25% continuous saponin-containing diet throughout grew better than those that were put on the control diet, although the average weight difference from control group was not statically significant.



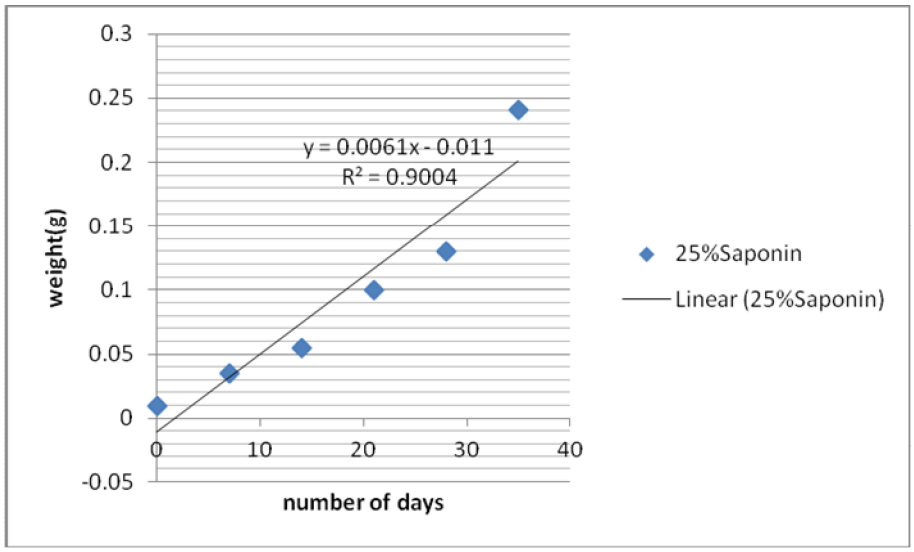
Figure(6:1) growth rate of female Nile tilapia fry durig the first five weeks treatment with 10%saponin and 25% saponin mixed with food compared to control.



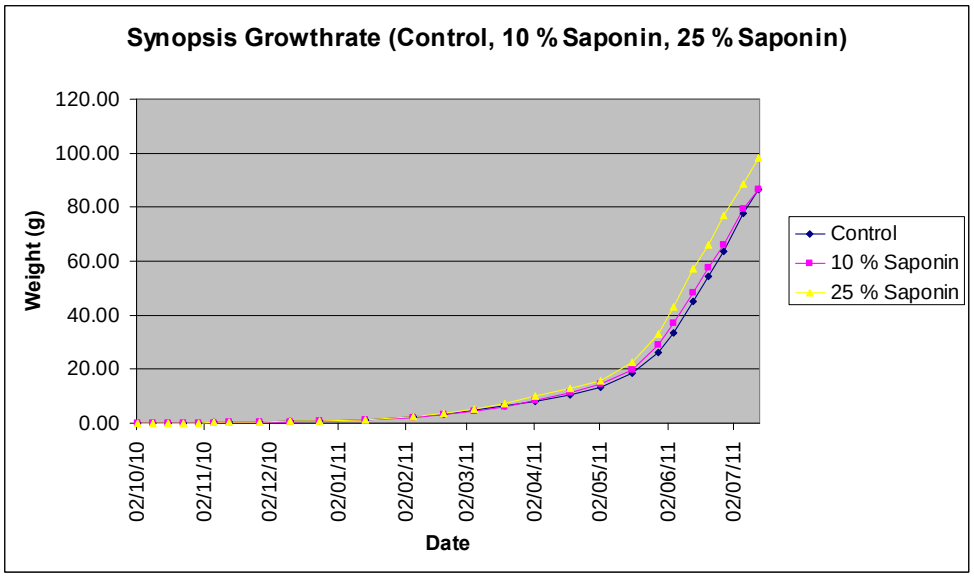
Figure(6:2): average growth rate of female Nile tilapia fry during (35days) experiment period in control group.



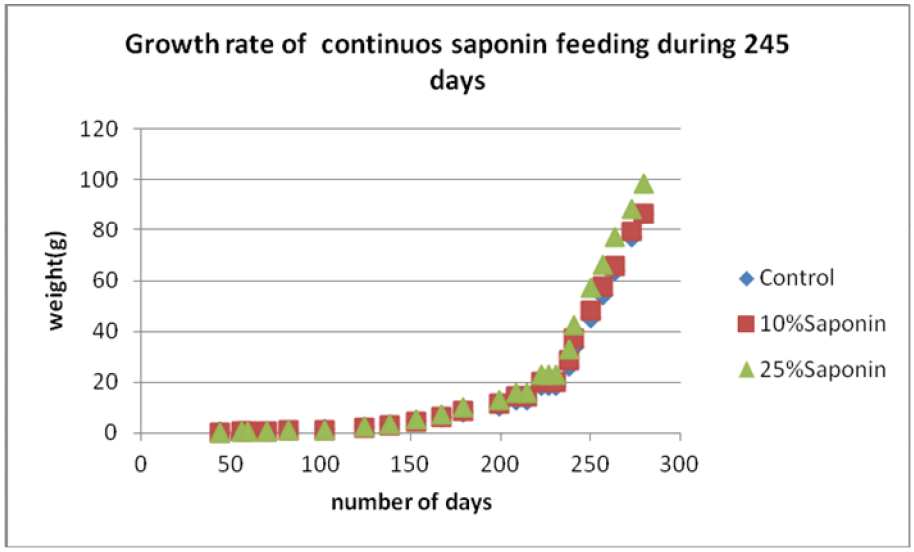
Figure(6:3): average growth rate of female Nile tilapia fry during (35days) experiment period in 10% saponin group.



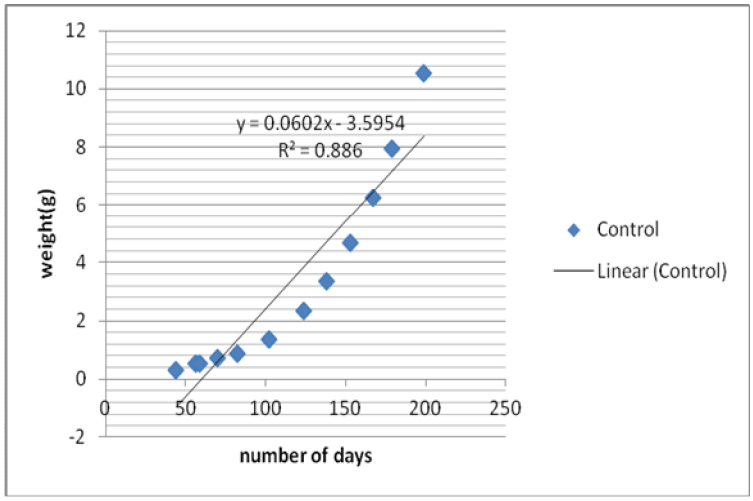
Figure(6:4) average growth rate of female Nile tilapia fry during (35days) experiment period in 25% saponin group



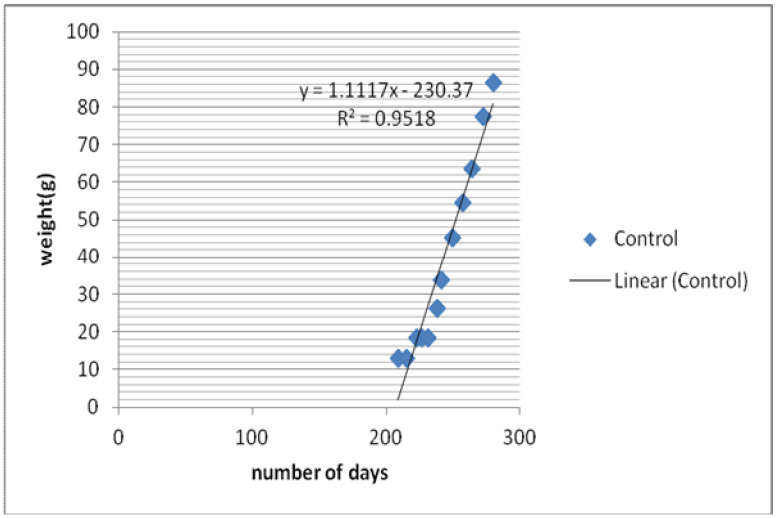
Figure(6:5) growth rate of female Nile tilapia fry durig 280 days treatment with continuous 10%saponin and continuous 25%saponin mixed with food compared to control.



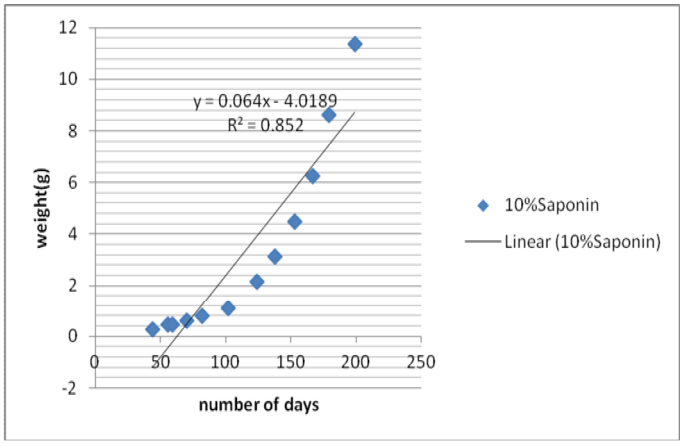
Figure(6:6) growth rate of female Nile tilapia fry durig 245 days treatment with continuous 10%saponin and continuous 25%saponin mixed with food compared to control.



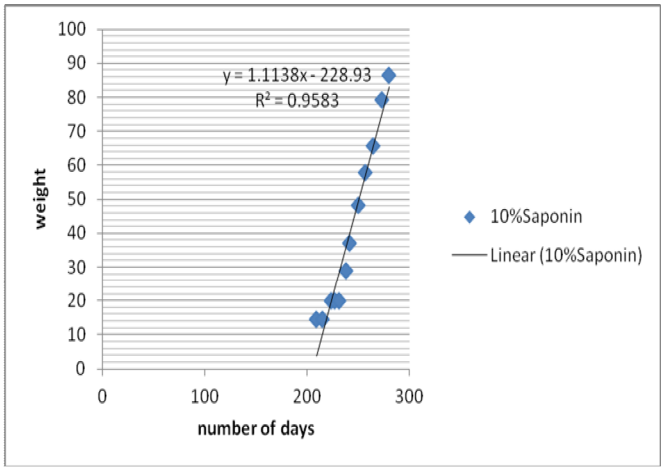
Figure(6:7): average growth rate of female Nile tilapia fry from(44-199days) experiment period in control group



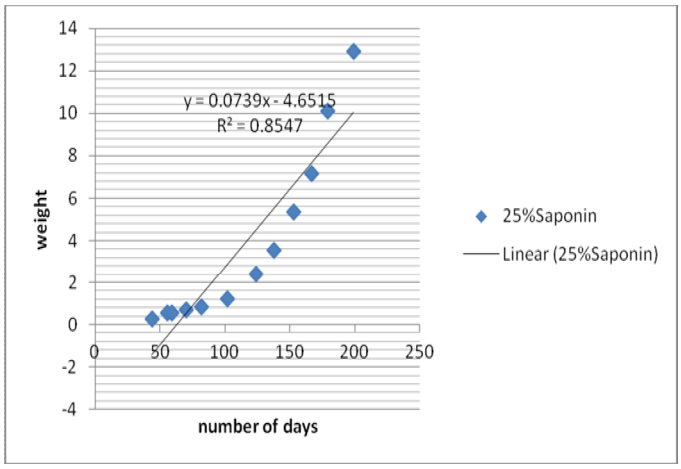
Figure(6:8): average growth rate of female Nile tilapia fry from(209-280days) experiment period in control group



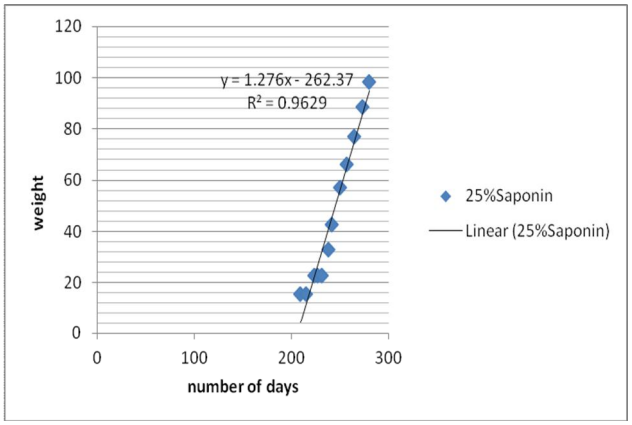
Figure(6:9): average growth rate of female Nile tilapia fry from (44-199days) experiment period in 10% saponin group.



Figure(6:10): average growth rate of female Nile tilapia fry from(209-280days) experiment period in 10% saponin group



Figure(6:11): average growth rate of female Nile tilapia fry from(44-199days) experiment period in 25% saponin group



Figure(6:12): average growth rate of female Nile tilapia fry from(209-280days) experiment period in 25% saponin group

Table 6.2: summary growth rates, average body weights, and food weight consumed of different treatments groups

	Control	10% Saponin	25% Saponin
Growth rate (0-35 days)	0.0058	0.0051	0.0061
Growth rate (44-199 days)	0.060	0.064	0.073
Growth rate (209-280 days)	1.11	1.11	1.27
Average weight (35 days)	0.096	0.085	0.095
Food weight consumed(g) (35 days)	0.554	0.488	0.534
Average weight (280 days)	86.60	86.45	98.35
Food weight consumed(g) (280 days)	149.5	153.4	175.3

Our results were consistence with previous studies, of (Francis et al , 2001b ,2002a,2002b) that showed that feeding Common carp (Cyprinus carpio L), 150mgkg−1 saponin in diets and 300mgkg−1to Niletilapia (Oreochromis niloticus L) produced higher rate of body mass gain. Our study showed that Fish that were fed on feed with 25% saponin in the first five weeks exhibited higher feed conversion efficiency FCE (0.45), and lower feed conversion ratio FCR (2.22) compared to control and fish fed with 10% saponin Table (6.1). On the other hand, fish fed with continuous 25% saponin over long period (280) days had feed conversion ratio FCR comparable to control and fish fed with 10% continuous saponin but the average final body weight but the average final body weight of fish tended to be higher than controls, although not statically significant ($p > 0.05$). This was in accordance with previous Bangladesh field study that showed no statistical significance difference in SGR between the different groups after the inclusion of 150 ppm QS ,500 ppm QS and 2000 ppm QS in mixed population tilapia diet for 25 days (Steinbronn, 2002) . On the other hand, the food conversion ratio (FCR) was lower in carp fed a diet containing150 mgkg−1 (0.82±0.07) and tilapia fed 300 mg kg−1 (1.4±0.19) of QS for 56 days compared to the respective controls (0.89±0.04and1.61±0.34) (Francis et al., 2001b,2002a). The lack of homogeneity in the growth-promoting effects of dietary QS both in the laboratory and field trials could reflect that growth promoting effects of QS is more pronounced during the initial period of feeding. Experiments gave some indications that the age and initial body

size of the fish may have an effect on the growth-promoting action of dietary saponins in carp (Francis et al , 2005).We do not know if sex-dependent differences exist. (Bosler et al, 1997) found that both male and female lambs fed up to40 mg Quillaja saponin /kg mixed with a basal diet had significantly higher average daily weight gains than controls but that the difference in weight gain was lower in the females. Further studies to emphasize this point are needed.

According to previous studies suggested mechanisms contributing to the growth promoting effects of quillaja saponin (QS) include an increase in the permeability of intestinal membranes to digested dietary components (Francis et al., 2002d) and/or a stimulation of the activity of digestive enzymes, which increases the efficiency of feed utilization. Dietary QS significantly increased the activity of gut enzymes, amylase and trypsin and liver enzymes lactate dehydrogenase (LDH) and cytochrome c-oxidase (CO) (Serrano et al , 1998). Saponins are known to influence the permeability of biological membranes by increasing their fluidity thus leading to changes in protein activities stimulating the activity of gut and liver enzymes. Saponins exert their biological activity either directly by influencing membrane permeability or systemically by absorption into blood stream or its degradation products (Francis et al, 2001b,2002a).

In the scope of this study suggested proposed explanation to our results will cover the following points: a- QS immune stimulant effect, b- bile acids-saponins interactions decreasing saponin systemic absorption c- type of saponin fractions, eluted with different methanol concentrations e-Salting out and decreased systemic availability.

Saponin have been reported to enhance the innate immunity in fishes (Harikrishnan et al, 2011). They induce the production of cytokines such as interleukins and interferons that might mediate their immuno stimulant effects (Kensil,1996). Oral administration of Quil A saponin increased leucocyte migration in yellowtail (Harikrishnan et al, 2011). Immuno stimulant effects of QS could decrease mortality, by facilitating better fighting of infections and improving feed utilization. Further studies are needed to confirm this fact. As we mentioned earlier the study was carried out in aquaculture ponds with approximate salinity of 4 TDS located at Jericho region in the Palestinian Authority. Intestinal water absorption in teleost fish (and likely other marine animals required to drink seawater), is critical for water balance and occurs against osmotic gradient as a consequence of Na+ and Cl transport, across epithelium in the whole length of the GIT including the gallbladder (Grosell et al,2006). External osmoregulatory sites in fish across leaky epithelia, include

the gall bladder, the renal tubule and small intestine in addition to gills. Bilea cids are also suspected of affecting salt and water transport, reducing in vitro Na+ and Cl transport as well as in vivo water and Na+ transport (Grosell et al,2011). Bile acid release is controlled by hormonal regulation, feed composition and environmental stress (Early et al,2003).It is well documented the indirect hypocholesterolaemic effect of saponins that interact with bile acids diverting them from enterohepatic cycle and increasing faecal excretion of bile acids-saponin complexes (Sidhu and Akefull 1986).We propose that increasing feed digestion and permeability by QS in addition to environmental stress osmoregulation will stimulate more bile acid secretion that will precipitate the QS diverting them from systemic absorption. Thus higher doses and more prolonged exposures for adequate systemic effect are critical. Furthermore we propose that QS increases gut permeability increasing water and NaCl transport across the intestinal membrane. A fact that could increase the salting out of sapoins precipitating them and diverting from systemic circulation. Salting-out describes the precipi-tation of a less soluble material from a solution in which it is mixed with other substances. Unfavorable orientation of water dipoles toward the less polar solute increases its salting out(Grover and Rayall,2004). Saponins are glycoside compounds whose chemical structures are composed of a fat-soluble nucleus called the aglycone that is a either triterpenoid (C-30), or neutral or alkaloid steroids (C-27) . One or more sugar side chains called glycones can be linked through ether and ester linkages to the aglycone nucleus at glycosylation sites. Triterpenoid saponins differ in sugar side chain structure and position (R1 to R5) on the aglycone nucleus. Triterpenoid saponins naturally occur as saponin or free aglycone forms. Quillaja are triterpenoid saponins with two sugar side chains at C-3 and C-28. It 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). Aqueous solubility are consistent with the expected interaction of the functional groups with the polar environment which is dependent on the number and orientation of hydroxyl groups. In aqueous solution, the activity coefficients decreased with increasing number of hydroxyl groups on the steroids(Cai et al,1997).

In the study of (Francis et al., 2001), the fish in the 300 mg/Kg QS treated group, especially the females, had significantly higher levels of muscle cholesterol, which was attributed to to a redistribution of nutrients caused by the suppression of spawning in the female fish or de-novo synthesis of cholesterol since (Chavali et al,1987)mentioned the possibility that saponins may cause increased denovo synthesis of cholesterol in mouse

spleen cells in vitro. Anabolic effect of saponins derived from plants preparations Ekdisten and Prime Plu originating from Leuzea rhaponticum sp causing elevated muscle mass was reported (Gadzhieva et al,1995).In accordance, continuous use of saponins could act as anabolic hormone like methyl testosterone without altering the reproductive performance as we will discuss later.

The study of (Stadtlander et al., 2010), showed that expression of growth hormone (GH) in brain and pituitary was highest for fish fed with 300 mg/kg of 60% trigonella saponin (60TS300) followed by control, while the other saponin fed groups showed a significantly reduced expression of GH such as 80TS300 and 60TS600. Fish fed other saponin (600 mg/kg of 60%) fractions tended to have inferior performance. Fish fed with 60TS300 grew numerically best in terms of body mass gain and final body mass compared to all other groups. In addition, expression levels of GH did strongly correlate to growth related parameters like body mass gain (BMG) ($r = 0.99$, $p < 0.01$), specific growth rate (SGR) ($r = 0.99$, $p < 0.001$), nutrient utilization parameters like food conversion ratio (FCR) ($r = -0.98$, $p < 0.005$), and energy retention (ER) ($r = 0.96$, $p < 0.01$). Thus high expression levels of GH and IGF-1 genes resulted in numerically highest growth rates and best nutrient utilization while significantly reduced gene expressions of GH and IGF-1 resulted in numerically lowest growth and inferior nutrient utilization.

In our study, fish that were fed on feed with 25% saponin in the first five weeks (critical period) exhibited significantly higher body weight gain percentage (BWG)% (2307), specific growth rate percentage SGR% (9), and lower feed conversion ratio FCR (2.22) compared to control and fish fed with 10% saponin. table(6.1), table(6.4), figs (6:1, 6:4). This could indicate, that saponin may acts as growth enhancer during the critical period, affecting growth hormone expression, as mentioned above, since growth hormone is very important during early stages of development. Fish fed with 10% Saponin showed lower growth rate GR compared to control and 25% Saponin group. Figs (6:1, 6:3), table (6.4). Thus, feeding 10% Saponin did not affect growth and optimum dose for growth enhancement is to feed fish starting from25% Saponin. On the other hand fish fed with continuous 25% saponin over long period (280) days gave higher percent body weight gain (BWG)% (983409), but feed conversion ratio FCR were comparable to control and fish fed with10% continuous saponin. Table (6.1). Additionally, no significant differences were found in growth rate (GR) between different groups during continuous saponin feeding figs (6:5, 6:6, 6:7, 6:8, 6:9, 6:10, 6:11, 6:12), table (6.4). Continuous saponin feeding did not

affect FCR, and GRs parameters, but average body weight, and BWG% were higher with 25% continuous saponin group. This fact could be contributed to saponins effect on intestinal digestion and absorption or adaptation to continuous intake of QS along with the diet as mentioned by Francis or attributed to other mechanisms that limit its availability to systemic circulation.

6.3 Sex ratio

There was no proportion of males and females in all groups. All the fish were female and they put eggs. There was no effect of Quillaja saponin on sex inversion. The study of (Francis et al ,2002c) reported significant deviation of sex ratio in tilapia larvae fed continuously over a 6-month experimental period a diet containing 700 mg QS kg−1from the normal 50:50 ratio in favor of males. This was also evident (but not statistically significant) in the treatment groups receiving lower quantities of QS 150mg kg−1. Bangladesh, field experiments failed to confirm the laboratory results on the effects of dietary QS on sex ratio. The sex ratios in tilapia larvae fed diets containing 150 and 500 ppm QS which produced offspring did not differ significantly from 50:50 ratio for males and females, but the proportion of males was slightly higher in both treatments as compared to the control group. On the other hand no reproduction was observed in the fish supplemented with highest level 2000 ppm QS with more female than male tilapia larvae fed and 20%mortality during the early rearing phase. Production of fry was completely suppressed even after the removal of saponins from the diets in this group (Steinbronn, 2002). Reproduction was not suppressed in our experiment with no sex-inversion which amplify that probably higher doses are needed to suppress fertility and to produce sex inversion. On the other hand the use of very high doses to alter sex ratio is economically insignificant due to the high cost. This is the first study demonstrating the QS growth promoting effect at very low concentrations (2ppt)and could establish the minimal concentration eliciting growth promoting effect which is 2ppt of the 25% saponin. It is obvious that QS can control tilapia reproduction, but at high concentrations which could limits its use from the economic point of view.

Administration of exogenous synthetic androgens in the feed for the crucial period of sexual differentiation is the most popular method for producing monosex populations in fish (Devlin and Nagahama, 2002). The most widely used androgen is methyltestosterone (MT), which probably exerts its masculinization activity through the activation of the

androgen receptor. Saponins are steroidal or triterpenoidal glycosides, with similar structure to methyltestosterone (MT) common in a large number of plants and plant products that are important in both human and animal nutrition. Saponins have been found to significantly affect growth, feed intake and reproduction in animals. Some are inhibitory and other are stimulatory (Das et al,2012).

Failure of QS-sigma preparations to suppress fertility in our experiment could be attributed to the following: a- type of saponin fractions, eluted with different methanol concentrations and biological activity b- higher doses are needed to produce the desired effect as discussed above, c- aromatase theory and stimulation of LH release by Saponin ,in addition to the effect of water salinity on LH release and reproduction, d-decreased availability of saponins to systemic circulation in order to elicit their systemic effects as we discussed earlier in growth section.

Evidence exists that the biological activity of saponins is not the consequence of one single biologically active saponin or sapogenin which can be extracted and purified but rather that saponin mixtures exhibit the highest biological activity. Biological activity depends on the nature of the saponins on one side and the degree of saponin fractionation on the other side. It was observed that the stereochemistry of the terminal sugar on the saccharide chain appears to be important in conferring activity on the saponin molecule because of its ability to affect the overall shape of the molecule (Gee et. al. 1998).Increased or decreased saccharide branching affects permeabilising activity of saponins. For example, De-acylated Quillaja saponins1and2,which differ only in the absence of one glucose residue, differed significantly in their ability to stimulate absorption of insulin despite having similar surfactant strength and haemolytic potency(Pillion et al 1996).This is probably due to its interaction with specific receptors at the membrane level which is dependent on the binding site that may be is altered by structural changes. Studies of the effects of three triterpenoidal saponin preparations from Quillaja saponaria, soybean, and Gypsophila paniculata on cell permeation showed that each exhibited an individual mode of action attributed to side chain structures and the extend of membranes rearrangement (Levavi-Sivan et al, 2005).

In general isolation of Saponins begin with the extraction of the plant material with aqueous methanol or ethanol. Further processing of the extract is carried out after evaporation under reduced pressure, dissolution in a small amount of water and phase separation inton butanol. Further purification is then carried out using HPLC separation.

Usually certain of the above steps have to be repeated with a change of support or eluent to achieve high purity. In a previous experiment by (Stadtlander *et al*., 2008) to study the effect of saponin in mixed sex tilapia populations, saponins were extracted by the conventional Soxhlet method with hexane/ethanol and fractionated and isolated by consecutive methanol concentrations of 40, 60 and 80% (Trigonella foenum-graecum,TS) and 80% (Quillaja saponaria, QS). Extracted saponins were added to the diets of Nile tilapia larvae in two different concentrations, 150 and 1000 ppm. Results showed that the percentage of non-females ranged from 52% (40% TS, 150 ppm) to 73% (80% QS, 150 ppm) with two treatments (80% TS 150 ppm and 60% TS 150 ppm) being significantly different ($p < 0.05$) from the expected 50:50 ratio.

In our experiment, the treated fish tended to have an enhanced food conversion compared to control. However, Quillaja saponin sigma preparations did not affect tilapia fertility. Previous studies showed that masculinisation effect is dependent on the type of saponin fractions, eluted with different methanol concentrations, and dose . In the current experiment the 25% saponin used is more refined and highly pure with greater biological activity than the 10% saponin. Thus it is clearly obvious that Quillaja 10%saponin sigma preparations neither served as aromatase nor as growth enhancers. Higher doses of Quillaja 25% saponin sigma preparations could be required to achieve sex-inversion or it did not show the potential to serve as aromatase inhibitors. Since its use at very high doses is very expensive and our aim in aquaculture is to increase nile tilapia productivity using natural plant products with minimal cost, it is recommended to be used as growth enhancers with no role in sex inversion.

(Golan et al., 2008) demonstrated that Quillaja saponins inhibit tilapia aromatase activity in vitro with highest potent concentration in the 80% ethanol eluate . Aromatase inhibitors work by inhibiting the action of the enzyme aromatase, which converts androgens into estrogens by a process called aromatization. Expression of ovarian aromatase in Nile tilapia lay between 3 and 4 days post fertilization in both sexes, with levels of expression high in females that play a decisive role in sexual differentiation achieved by down-regulation of the expression of this gene in males (Kwon et al 2001). In-vivo studies also showed deviation from the normal 50:50 male to female ratio although it was statiscally non significance as mentioned earlier. Bangaladish study failed to confirm sex inversion,but very high saponin concentrations 2000 ppm suppressed firtility completely.Further more QS was found to stimulate LH release from dispersed tilapia

pituitary cells in vitro but did not show any diet-dependent (QS-containing or control diets) trend in either male or female tilapia in vivo (Francisetal,2002d). Acording to aromatase theory We propose that alteration of aromatase activity by giving Qs during the crucial period decreases the rate of aromatization which alters E2 (estrogen) levels but not sufficient to cause complete sex inversion. QSs inhibited aromatase but may be with lower potency in vivo than the non-selective aromataze inhibitors like fedazole (Kwon et al, 2000).This is clear from the lack of homogeneity in the sex-inversion ratios of dietary QS both in the laboratory and field trials at different concentrations. Low (E2) release the feedback inhibition on gonadotropins producing high FSH levels that inhibits ovulation, since it is present in the blood of immature fish and levels increase during the vitellogenic phase but should decline towards follicular maturation and spawning (Kawauchi et al 1989).Continous use of Qs there after could stimulate LH release from the pituitary. Release of LH from the pituitary is important for triggering the process of ovulation.It was found that P. macrocarpus saponins stimulated the LH release in a dose-dependent manner from 10-mu-g/ml to 300-mu-g/ml. Scanning electron microscopy did not revealed any significant alteration of the cell structure at low concentrations of saponins of 10-mu-g/ml, and membrane destortion was only at high concentrations(EL Izzi et al,1992). Dose dependent leakage of Luteinizing Hormone (LH;MW35,000) from tilapia pituitary dispersed cells with Qs was also reported by (Levavi-Sivan et al,2005). Additional studies emphasazing the effect of QS on LH release in vivo are required.

We earlier pointed to the salinity of Jericho ponds used in our experiments which is 4TDS. (Watanabe and Kuo,1985) found that the total number of spawnings of Nile tilapia females was greater in brackish water rainging from(5-15%) than in either full strength seawater (32%) or fresh water.It was also shown that water hardness and water-salinity affected the spawning rates of O. niloticus females (Stamer 2001).(Greer et al,1985) demonstrated that Perifusion of human adenohypophyseal cells with hypertonic medium depressed LH secretion; return to isotonicity caused an immediate high-amplitude “off” burst of LH secretion closely resembling that induced by hypotonic perfusion. We preposes that changes in plasma osmotic pressure after fish transfer from fresh to brakish water could stimulate LH secretion. Further studies are evaluating the effect of salinity on LH release are needed.

Chapter Seven

Conclusion and Recommendations

In our experiment, fish that were fed on feed with 25% saponin in the first five weeks (critical period) exhibited significantly higher body weight gain percentage (BWG)%, specific growth rate percentage (SGR) %, and lower feed conversion ratio FCR compared to control and fish fed with 10% saponin. On the other hand no significant differences were found in growth rate (GR) between different groups during continuous saponin feeding. Continuous saponin feeding did not affect FCR, and GRs parameters, but average body weight, and BWG% were higher with 25% continuous saponin group, which could be contributed to saponins effect on intestinal digestion and absorption or attributed to other mechanisms that limit its availability to systemic circulation.

Previous studies showed that masculinisation effect is dependent on the type of saponin fractions, eluted with different methanol concentrations, and dose.

In the current experiment the 25% saponin used is more refined and highly pure with greater biological activity than the 10% saponin. Thus it is clearly obvious that Quillaja 10% saponin sigma preparations neither served as aromatase inhibitors nor as growth enhancers. Higher doses of Quillaja 25% saponin sigma preparations could be required to achieve sex-inversion or it did not show the potential to serve as aromatase inhibitors. Since its use at very high doses is very expensive and our aim in aquaculture is to increase nile tilapia productivity using natural plant products with low input costs, it is recommended to be used as growth enhancers with no role in sex inversion.

Recomendations

-Future research should concentrate on understanding the physiological mechanisms by which dietary saponins increase growth and feed conversion efficiency in tilapia.

-Further experiments are needed to assess the impact of different levels of Quillaja saponins on growth and reproduction of tilapia and to study the mechanism of action.

- Because flow through system consumes much water, it is much better to use the discharge water for agricultural.

-If it is possible, It is better to grew Quillaja Saponaria in order to extract saponin to make it available and cheaper.

- Future researches have to examine the relationship between our experimental saponin concentrations and the growth hormone.

- The use of sigma QS preparations at high doses to induce sex inversion is not recommended. Since our aim in aquaculture is to increase nile tilapia productivity using natural plant products with low input costs, it is recommended to be used only as growth enhancers with no role in sex inversion.

- Since the minimal concentrations eliciting growth promoting effect was established, using it at low doses is more economic efficient and reduces laborious work of extraction from plant sources.

- Since the saponin gave better growth rate and enhanced the growth, there could be a feeding strategy to combine it with fish feed. Also, Future research have to examine the effect of different saponin types and saponin concentrations, in order to know the effect on fish growth and sex inversion.

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Appendix:

Table 1: Fish average body weights

Date	Control (g)	10 % Saponin Continuous feed (g)	25 % Saponin Continuous feed (g)
02/10/10	0.01	0.01	0.01
09/10/10	0.04	0.03	0.04
16/10/10	0.06	0.05	0.05
23/10/10	0.10	0.09	0.10
30/10/10	0.15	0.13	0.13
06/11/10	0.22	0.20	0.24
13/11/10	0.30	0.26	0.32
27/11/10	0.54	0.47	0.57
11/12/10	0.72	0.60	0.68
24/12/10	0.88	0.81	0.81
14/01/11	1.37	1.08	1.22
05/02/11	2.35	2.14	2.40
19/02/11	3.36	3.12	3.57
05/03/11	4.68	4.50	5.34
19/03/11	6.26	6.24	7.18
02/04/11	7.93	8.63	10.11
18/04/11	10.54	11.37	12.89
02/05/11	13.09	14.43	15.71
16/05/11	18.37	19.85	22.69
28/05/11	26.16	28.81	33.05
04/06/11	33.57	36.94	42.94
13/06/11	45.12	48.18	56.98
20/06/11	54.43	57.75	66.12
27/06/11	63.61	65.85	76.94
06/07/11	77.52	79.34	88.56
13/07/11	86.60	86.45	98.35

Table 2: Average food weight consumed

Date	Control	10 % Saponin	25 % Saponin
9-10-2010	0.0532	0.0462	0.049
16-10-2010	0.0861	0.0742	0.0763
23-10-2010	0.1029	0.098	0.1043
30-10-2010	0.1596	0.1323	0.1365
6-11-2010	0.15274	0.1379	0.16849
15-11-2010	0.39996	0.35433	0.35433
27-11-2010	0.6492	0.5676	0.6864
30-11-2010	0.1299	0.1134	0.1374
11-12-2010	0.7887	0.6622	0.7469
24-12-2010	1.1284	1.0608	1.05794
14-1-2011	2.738	2.162	2.44
5-2-2011	3.4482	3.1521	3.528
19-2-2011	3.29	3.0576	3.4958
5-3-2011	3.5085	3.3735	4.0035
19-3-2001	3.5056	3.4944	4.0208
2-4-2011	1.9044	2.07	2.4264
22-4-2011	7.22	6.822	7.736
2-5-2011	3.273	3.609	3.928
8-5-2011	2.3568	2.598	2.8284
16-5-2011	4.4088	4.764	5.4464
20-5-2011	2.9388	3.176	3.6308
24-5-2011	3.3064	3.5728	4.0844
1-6-2011	7.3248	8.0682	9.2547
4-6-2011	4.0524	4.4325	5.1117
13-6-2011	16.2423	17.3448	20.5137

Table 2 Continued

Date	Control	10 % Saponin	25 % Saponin
20-6-2011	15.2397	16.17	18.5129
27-6-2011	15.5855	16.1322	18.8489
6-7-2011	24.3477	24.9912	27.8973
13-7-2011	21.2177	21.1806	24.0961
Average feed (280days)	149.5593	153.4178	175.3214

أثر مادة الصابونين المستخرج من نبتة الكلاجة على نمو وسلوك سمك المشط

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ملخَّص

أُجريت الدراسة الحالية في مختبر المسطحات المائية في جامعة القدس من بداية شهر أكتوبر 2010 وحتى شهر حزيران 2011 بحيث تم وضع 750 سمكة بعمر تسعة أيام في نظام مخصص لدخول الماء إلى الأحواض السمكية وخروجه من خلالها ليحافظ على بيئة مناسبة ونظيفة لنمو الأسماك من خلال إخراج الفضلات والغذاء المتبقي والأمونيوم والنيترايت، وزّعت الأسماك على 25 حوض بحيث تم تقسيمها إلى خمس مجموعات، مجموعة A: المجموعة الضابطة، مجموعة B: غذاء عادي مع صابونين 10% لمدة 5 أسابيع، مجموعة C: غذاء عادي مع 10% صابونين بشكل مستمر، مجموعة D: غذاء عادي مع 25% صابونين لمدة 5 أسابيع، مجموعة E: غذاء عادي مع 25% صابونين بشكل مستمر. بعد نهاية الأسبوع الخامس تم نقل الأسماك الى برك صناعية في مدينة أريحا بحيث تم دمج مجموعة B و مجموعة D مع المجموعة الضابطة وأصبحت مجموعة واحدة تتناول الغذاء العادي الخالي من الصابونين مع الحفاظ على فصل المجموعات عن بعضها. كان يتم وزن السمك بشكل أسبوعي وذلك من أجل حساب كمية الغذاء اللازم تقديمه للأسماك و معرفة معدل النمو، كانت كمية الغذاء المقدمة للأسماك تعتمد على وزن السمكة. خلصت الدراسة إلى وجود فروق دالّة احصائياً في نمو الأسماك حسب اختلاف نوع الغذاء المتناول، حيث أن الأسماك التي تناولت الغذاء الممزوج مع 25% صابونين في الأسابيع الخمس الأولى كانت تملك أكبر وزن، أكبر استفادة من الغذاء، أكبر كفاءة في تحويل الغذاء وأعلى معدل نمو بالمقارنة مع 10% و المجموعة الضابطة، حيث أن هذا التركيز أثر على معدل النمو في المراحل الأولى من عمر السمكة. أما على المدى الطويل أي اعطاء الصابونين بشكل مستمر، فلم يكن هنالك فروق ذات دلالة إحصائية من حيث

معدل النمو أو كفاءة تحويل الغذاء أو الاستفادة من الغذاء. بالرغم من ذلك كانت مجموعة 25% تملك أكبر معدل نمو، أكبر وزن وأكبر نسبة استفادة من الغذاء مما قد يتم تفسيره على أن الصابونين زاد من عملية هضم الطعام وامتصاصه أو ساهم من ناحية أخرى في تقليل وجوده في الدم. وبالنسبة لوجود الذكور فلم يوجد ذكور فكلها كانت إناثاً. أما بالنسبة لتركيز 10% فلم يؤثر في نمو السمكة أو حتى في قلب الجنس وتثبيط إفراز هرمون الأروماتيز. وبالنسبة لجنس السمك فلم يحدث أي تغيير أو قلب في جنسها فكلها وضعت بيوضاً، فلم يكن هنالك أثر لمادة الكلّاجة بهذه التراكيز على تغيير جنس سمك المشط وبالتالي لم تؤثر على نشاط أنزيم الأروماتيز المسؤول عن انتاج هرمون الإستروجين الأنثوي. وهذا يعطي مؤشراً إلى الحاجة لتركيز أكبر من 25% صابونين لقلب جنس السمك من إناث إلى ذكور. وبما أن استخدام مادة الكلاجة بتراكيز مرتفعة لإحداث خلخلة بالتكاثر غير مجدٍ اقتصادياً فإنه ينصح فقط باستخدامها كمحفز للنمو وليس لها أي دور في قلب الجنس بناء على نتائج البحث.