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Dietary Administration of Plant Extracts for Production of Monosex Tilapia: Searching A Suitable Alternative to Synthetic Steroids in Tilapia Culture

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Abstract

Study was aimed to evaluate the efficacy of *Mucuna pruriens* seeds and *Asparagus racemosus* roots for induction of masculinisation in tilapia. Plant materials were extracted with water, ethanol, methanol and successive methanol and mixed sex juveniles of Nile tilapia were subjected to dietary treatment with the extracts at the concentration of 0.1, 0.15 and 0.2 g/kg feed. All treatments produced significantly higher ($P < 0.05$) percentage of males than control. The highest percentage of males (93.79 ± 0.95) was obtained by treatment with methanol extract of *M. pruriens* seeds at the concentration of 0.2 g/kg feed. For *A. racemosus* roots also, the highest percentage of males (92.24 ± 0.13) was obtained with methanol extract at the concentration of 0.2 g/kg feed. Extracts with different solvents of both the plants showed presence of steroid/terpenoid, saponin and flavonoid. The highest percentage of phenol (658 mg of GAE/g dw) and flavonoid (11.72 mg of RE/g dw) content for *M. pruriens* was found in aqueous extract whereas for *A. racemosus* in successive (629 mg of GAE/g dw) and ethanolic extracts (22.08 mg of RE/g dw) respectively. The antiradical activity is highest in methanolic extract of *Mucuna* (18.265%) and ethanolic extract of *Asparagus* (98.522%).

Keywords: Antiradical activity; *Asparagus racemosus*; *Mucuna pruriens*; Phytochemicals; Sex reversal

Introduction

The Nile tilapia, *Oreochromis niloticus* (Linnaeus) is currently ranked second only to carps in global production and is likely to be the most important cultured fish in the 21st century (Ridha, 2006). Rapid growth, high tolerance to low water quality, efficient food conversion, resistance to disease, ease of spawning and good consumer acceptance make tilapia a suitable fish for culture (El-Saidy & Gaber, 2005). Females of tilapine species have a high fecundity, generally reproducing at a small size and exhibiting stunted somatic growth at higher densities, while male tilapias exhibit faster growth rates and are often the preferred gender for monosex aquaculture (Hines & Watts, 1995). Use

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of synthetic steroids for production of monosex all-male tilapia is often associated with different ecological and health-related hazards, and hence natural compounds may be explored as a potential alternative in this regard (Papoulias *et al*, 2000). Plant extracts containing diverse bioactive principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils have been reported to promote various activities like antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and antimicrobial properties in fish culture (Citarasu, 2010; Chakraborty *et al*, 2011). Phytochemicals are also reported to block biosynthesis as well as action of estrogen by acting as aromatase inhibitors and antagonists to nuclear estrogen receptor in gonad germ cells (Rempel *et al*, 2008) and hence may be considered as potential mean for inducing sex reversal in fish. However, there are significant variations regarding the efficacy of different phytochemicals for production of all-male fish population and the potential anabolizing and virilizing effects of such plant extracts needs to be clearly documented.

The herb, *Mucuna pruriens* (Linnaeus) has various therapeutic uses and aphrodisiac effects in mammals (Suresh *et al*, 2010). It has been reported to possess medicinal values and some works indicated the potential use of *M. pruriens* as alternative source of protein in fish feed (Ignacimuthu *et al*, 2008, Pugalenthi *et al*, 2005, Sridhar *et al*, 2007). The plant has been found to increase libido in men due to its dopaminergic properties (Giuliano *et al*, 2001, Shukla *et al*, 2010). Treatment with ethanolic extracts of *M. pruriens* seed has resulted a significant and sustained increase in sexual activity, improved mount, intromission and ejaculation, and decreased latencies in normal male rats (Suresh *et al*, 2009, Suresh *et al*, 2010). However, no studies have been reported related to its *in vivo* effect on sex reversal, growth and immunostimulation of fish. The plant, *Asparagus racemosus* (Linnaeus) has also been reported to have medicinal values, various therapeutic uses and aphrodisiac effects in mammals (Thakur *et al*, 2009; Mishra *et al*, 2010; Alok *et al*, 2013). It was found to stimulate growth in fish as well (Borkar *et al*, 2014). But, use of these two plant extracts for sex reversal and growth induction in tilapia during its culture under Indian perspective is not documented. The type and amount of phytoconstituents in the plant extracts may vary with different solvents used for extraction, thereby showing variable results with respect to induction of masculinity (Tiwari *et al*, 2011). Therapeutic properties of plant extracts are often correlated with the presence of phenolics and flavonoids in the extract (Sharma *et al*, 2014). Considering these aspects, the objective of the present study was to investigate the potential effect of these two plants on the masculinization of *O. niloticus*, to find the most potent solvent for extraction of the phytochemicals from the two plants that would yield the highest androgenic action and to determine an ideal treatment regime for each plants that might produce maximum percentage of males in tilapia. The phenol and flavonoid content of the extracts and their interrelationship with the antioxidant activity of the respective extracts were also determined.

Materials and Methods

Collection of Fish Seed

Just hatched juveniles of mixed-sex *O. niloticus* were collected from the Fish Hatchery of West Bengal Government, oxygen packed and transported to the laboratory.

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Plant Extracts Preparation

M. pruriens seeds and *A. racemosus* roots were procured from the local plant market, washed in sterile distilled water, air-dried in shade and powdered. These powdered plant materials (250 g) were extracted with 500 ml solvents such as water, methanol, and ethanol in a percolator and the extracts were evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored at -20ºC in amber glass bottle until those were used (Hussain *et al*, 2009). For successive methanol extraction, plant powders (200 g) were subjected to extraction by maceration under gentle agitation in a glass vessel for 48 h at room temperature using successively hexane (200 ml for 5 h, three times), dichloromethane (200 ml for 5 h, three times) and methanol (200 ml for 5 h, three times) (Moundipa *et al*, 2005). The methanol extract was evaporated and stored at -20ºC.

Dietary Treatment with Different Solvent Extracts of the Plants

The treatment categories had 2×4×3 factorial design: the first factor was plant materials (*M. pruriens* seeds and *A. racemosus* roots), the second factor was related to solvents used for extraction (aqueous, methanol, ethanol and successive methanol), and the third factor was related to concentrations of extracts used for dietary treatment (0.1, 0.15 and 0.2 g/kg feed). Three days old mixed sex juveniles of Nile tilapia (n=3600; mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were randomly assigned in 5-liter glass aquaria (8 fish/liter; 40 fish/aquaria) and three aquaria were assigned for each treatment category. Besides, fish were also distributed in 18 more aquaria as control and those fish were fed diets fortified with no plant material. Plant extracts at desired concentrations were dissolved in dimethyl sulfoxide (DMSO) and added to finely ground (<500-1000 µm) artificial diet containing 30% crude protein (Tokyu, Japan) (Moundipa *et al*, 2005). The control feed was prepared by adding only DMSO to finely ground artificial diet. The feed was then wetted with deionized water, mixed thoroughly, formed into pellets with a pelleter (diameter 2 mm), and dried at room temperature. Pelleted feed was pulverized before feeding to the juvenile fish and fish were fed respective diets twice daily at the rate of 20% body weight/day for 30 days. The aquaria were continuously aerated and maintained in heated (T = 27 ± 2ºC) static systems. Water in all aquaria was replaced daily and the fish was kept under similar photoperiod (14 L: 10 D).

Sexing of Fish

After 30 days, all the fish from each treatment group including control were anesthetized with phenoxy-ethanol (1:20000, v/v) and sacrificed. Sex ratio following each treatment was determined by macroscopic and microscopic examinations of gonad tissue using the standard acetocarmine squash technique (Guerrero & Shelton, 1974).

Qualitative Phytochemical Studies

Qualitative phytochemical analysis of different solvent extracts of *M. pruriens* seeds and *A. racemosus* roots were carried out using standard procedures (Malpani *et al*, 2011; Kumar & Bhardwaj, 2012; Ray *et al*, 2013).

Determination of Total Phenolic Content

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The total phenolic content of the plant extracts was determined by standard procedure using Folin-Ciocalteu reagent (Maisuthisakul *et al*, 2007). The absorbance of plant extracts and a prepared blank were measured at 765 nm with a spectrophotometer (UV–Vis model 1601, Shimadzu). The concentration of total phenolic compounds in all plant extracts was expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight of plant, using the linear Eq. (2) derived from Eq. (1), which was determined from known concentrations of gallic acid standard prepared similarly.

Absorbance (at 765nm) = Constant × Gallic acid concentration(1)

Gallic acid equivalents (GAE) = Absorbance (at 765nm) / 0.41(2)

Determination of Total Flavonoid Content

The total flavonoid content of plant extracts was evaluated by a colorimetric assay using standard protocol (Maisuthisakul *et al*, 2007). The absorbance was read immediately at 425 nm using a spectrophotometer (UV–Vis model 1601, Shimadzu, Kyoto, Japan). The absorbance of a prepared blank was also recorded. Total flavonoid content expressed as milligrams of rutin equivalents (RE) per gram dry weight of plant, using the linear Eq. (4) derived from Eq. (3), which was determined from known concentrations of rutin standard prepared similarly.

Absorbance (at 425nm) = Constant × Rutin concentration (3)

Rutin equivalents (RE) = Absorbance (at 425nm) / 4.71 (4)

Study of Antioxidant Properties of the Plant Extracts

The free radical scavenging activity of the plant extracts was evaluated using the stable radical DPPH by standard methods (Maisuthisakul *et al*, 2008). Absorbance was measured at 517 nm. The percentage of DPPH radical scavenging activity of each plant extract was calculated from $[A_0-(A_1-A_s)]/A_0 \times 100$. A_0 is the absorbance of the control solution (containing only DPPH); A_1 is the absorbance of the DPPH solution containing plant extract; and A_s is the absorbance of the sample extract solution without DPPH. The DPPH radical scavenging activity (%) was plotted against the plant extract concentration (mg/mL) to determine the concentration of extract necessary to decrease DPPH radical scavenging by 50% (EC_{50}). These values were changed to antiradical activity (AAR) defined as $1/EC_{50}$, since this parameter increases with antioxidant activity.

Statistical Analysis

Data were analyzed by IBM SPSS Statistics Version 20 software. Normality of variables was checked before conducting T-probe or ANOVA in GLM where solvent and concentration were considered as fixed and plant as random factors. Treatment means were compared by Tukey's HSD test for fixed factors. For variables not normally distributed nonparametric median tests were applied to evaluate treatment effects. All data are expressed in terms of mean ± standard error (SE).

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Results and Discussion

There was no significant difference (P>0.05) in survival percentage between fish fed plant extract fortified diets (89.27 ± 1.19%) and control diet (89.86 ± 2.70%). Such high survival for the treated fish indicated no adverse effects of treatment with both plant extracts on the general health of the fish. Similar results were obtained in other studies with catfish (*Clarias gariepinus*) and Swiss albino mice as well where oral administration of *M. pruriens* has no significant adverse effect on survival (Dada & Ogunduyile 2011, Okafor *et al*, 2013). In our previous experiment as well, immersion treatment with *A. racemosus* aqueous extract was found to have no adverse effect on general fish health (Mukherjee *et al*, 2015).

In control fish, the percentage of males was 43.48 ± 1.67 and the percentage of females was 56.52 ± 1.67 and no intersex fish was found. Treatment with plant extracts resulted in significantly higher (P<0.05) percentage of males (76.50 ± 1.30) compared to that of control. 19.10 ± 1.16% of treated fish was females, while 4.40 ± 0.45% treated fish was found to be intersex. However, the variables except the percentage of males in different treatment categories were not normally distributed and could not be transformed to achieve normal distribution.

In treatment with *M. pruriens* seeds, the percentage of males for every concentration differed significantly (P<0.05) from each other, and the highest percentage of males (82.82 ± 3.63) was observed at the concentration of 0.2 g/kg feed. In treatment with *A. racemosus* roots, no significant (P>0.05) difference was found in male percentage between the concentrations and the highest percentage of males (77.56 ± 2.94) was found at the concentration of 0.2 g/kg feed (Figure 1).

There was significant interaction effects (P<0.05) of plant material and solvent, plant material and concentration, solvent and concentration, and plant material, solvent and concentration for percentage of males (Table 1). Dietary administration of all the extracts from both plants at all different concentrations resulted in significantly higher (P<0.05) percentage of males compared to that in control fish (Table 1). For dietary administration of *M. pruriens* seeds, the highest percentage of males (93.79 ± 0.95) was obtained for treatment with methanol extract at the concentration of 0.2 g/kg feed and it was the highest among all the different treatment categories for both the plants (Table 1). In treatment with *A. racemosus* roots as well, the highest percentage of males (92.24 ± 0.13) was obtained with methanol extract at the concentration of 0.2 g/kg feed (Table 1). A dose-dependent increase in percentage of males was observed for all the solvent extracts of *M. pruriens* seed, while fish fed diets containing only methanol and successive methanol extracts of *A. racemosus* roots showed such dose dependent increase in percentage of males (Table 1). Such dose dependent masculinisation effect of plant extracts was also observed in other studies, where percentage of males increased with increase in the *Tribulus terrestris* concentration in *Poecilia latipinna*, *P. reticulata*, *Cichlasoma nigrofasciatum* and *Clarias gariepinus* (Kavitha & Subramanian, 2011; Kavitha *et al*, 2012; Çek *et al*, 2007a; Çek *et al*, 2007b; Turan & Çek, 2007). As the highest treatment concentration of 0.2 g/kg feed produced the maximum percentage of males for both the plants, further experiments with increased concentration might be required to achieve 100% sex reversal with these plants. Results of non-parametric tests for percentage of survival, females and intersex, which showed no normal distribution, indicated that only the medians of female

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percentage and intersex percentage differed significantly (P<0.05) across categories of solvent (Table 2). The ethanolic extract of *M. pruriens* seeds was found to significantly increase testosterone, LH, FSH and prolactin hormone levels, levator ani muscle weight, sperm count and motility in infertile obese mutant rat models (Kumar *et al*, 2011). Oral administration of *A. racemosus* roots showed pronounced anabolic effects such as significant weight gains in the body and reproductive organs, significant reduction of mount latency, ejaculation latency, post ejaculatory latency, intromission latency, and a considerable increase of mount frequency and penile erection in male albino rats (Thakur *et al*, 2009). Although the present work has indicated that treatment with extracts of different solvents of both the plants might induce high rate of masculinisation, whether this potency is caused by increase in androgen level cannot be deduced as the serum testosterone level was not measured during the study.

Qualitative analysis for phytochemicals revealed the presence of steroids in all the extracts of *M. pruriens* seeds and *A. racemosus* roots, which might render the androgenic activity of the extracts (Table 3). Flavonoids were found to be present in all the extracts, while tannins were present in aqueous, ethanol and methanol extracts for both the plants. Saponins were present in aqueous and ethanol extract of *M. pruriens* seeds and aqueous extract of *A. racemosus* roots. Alkaloids were present in methanol and successive methanol extracts of *M. pruriens* seeds, and successive methanol extract of *A. racemosus* roots, while glycosides were found in ethanol and methanol extracts of *M. pruriens* seeds and also in successive methanol extracts of *A.racemosus* roots. Carbohydrates were found in ethanol, methanol and successive methanol extract of *A. racemosus* roots and only in successive methanol extract for *M. pruriens* seeds (Table 3). A variety of pathways have been postulated to be associated with functional mechanisms of phyto-compounds causing both masculinisation and feminization at different concentrations (Chakraborty *et al*, 2014). Further investigations are required to deduce the functional mechanisms behind the androgenic potency of these two plants.

The ethanolic extract of *A. racemosus* showed the highest antiradical activity (98.522%) among all the solvents for both the plants, and in *M. pruriens*, methanolic extract was found to exhibit the highest percentage (18.265%) of antiradical activity (Table 4). The highest percentage (658 mg of GAE/g dry weight) of phenol content was observed in the aqueous extract of *M. pruriens* and in the methanolic and ethanolic extracts of *A. racemosus* (653 mg of GAE/g dry weight). The total flavonoid content for *M. pruriens* was observed to be the highest in aqueous extract (11.72 mg of RE/g dry weight), whereas for *A. racemosus* the ethanolic extract showed the highest total flavonoid content (22.08 mg of RE/g dry weight) (Table 4). Flavonoids, one of the many different phenolic phytoconstituents are reported to exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny, 2001, Yanishlieva-Maslarova, 2001). The high antiradical activity of *A. racemosus* ethanol extract might be associated with the high flavonoid content of the extract. Previous studies have reported weak correlation between antioxidant activity and total phenolics (Kähkonen *et al*, 2001; Velioglu *et al*, 1998). In the present study as well, weak correlations were observed between the antiradical activity and total phenol content (R^2 = 0.1107), and total phenolic and flavonoid contents (correlation coefficient, R^2 = 0.2978) for the extracts of *M. pruriens* and *A. racemosus* (Figure 2a, b). However, a higher correlation was observed between total flavonoid content and antiradical activity for the plant extracts (R^2 = 0.5804) (Figure 2c).

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Conclusion

The results emanating from this study might be implemented for development of eco-friendly aquaculture technique replacing synthetic hormones and chemotherapeutics with bio-degradable natural compounds. The result suggested that *M. pruriens* might be regarded to be more potent for induction of masculinization in Nile tilapia as it produced higher percentage of males compared to *A. racemosus*. Dietary administration of methanol extract of *M. pruriens* seeds at a concentration of 0.2 g/kg feed was found to be the best method for production of sex-reversed all-male tilapia. Presence of flavonoids in the plants indicated the health-promoting antioxidant activity of the plants as well. However, the highest percentage of males produced by the plant materials was found to be below the ideal requirement of 100% male population. Thus, further studies would be required to establish an ideal treatment regime for production of all-male tilapia population using the plant materials and to provide conclusive evidence regarding their efficacy to be used as a sex-reversal agent in tilapia culture. Studies are also warranted for identification and isolation of the plant bioactive compound responsible for the androgenic property for potential commercial use.

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Table 1. Percentage of males in tilapia fed diets containing extraction of *M. pruriens* seeds and *A. racemosus* roots with different solvents and at different concentrations.

Plant Material (P)	Solvent (S)	Concentration (C)	Male %
Control	-	0.00 g/kg	43.48±1.67^{a}
Mucuna pruriens Seeds	Aqueous	0.10 g/kg	78.30±0.87^{fghi}
		0.15 g/kg	81.52±0.77^{ghij}
		0.20 g/kg	89.37±0.47^{kl}
	Methanol	0.10 g/kg	81.81±1.00^{hij}
		0.15 g/kg	92.50±1.44^{l}
		0.20 g/kg	93.79±0.95^{l}
	Ethanol	0.10 g/kg	61.94±0.62^{c}
		0.15 g/kg	77.50±1.44^{fgh}
		0.20 g/kg	85.34±0.93^{jk}
	Successive	0.10 g/kg	52.13±1.32^{b}
		0.15 g/kg	61.07±2.32^{c}
		0.20 g/kg	62.77±1.43^{c}
Asparagus racemosus Roots	Aqueous	0.10 g/kg	77.30±1.46^{efgh}
		0.15 g/kg	73.00±1.14^{ef}
		0.20 g/kg	73.10±0.96^{ef}
	Methanol	0.10 g/kg	78.56±0.39^{fghij}
		0.15 g/kg	83.97±1.14^{hijk}
		0.20 g/kg	92.24±0.13^{l}
	Ethanol	0.10 g/kg	84.99±1.32^{ijk}
		0.15 g/kg	70.55±1.32^{de}
		0.20 g/kg	65.81±0.88^{cd}
	Successive	0.10 g/kg	63.82±0.93^{cd}
		0.15 g/kg	74.91±2.65^{efg}
		0.20 g/kg	79.09±0.67^{fghij}
PxS		S	
PxC		S	
SxC		S	
PxSxC		S	

Note: Different superscripts mark significant difference (P<0.05) in means within columns. S: Significant.

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Table 2. Non-parametric tests (Independent Samples Median Test) for survival percentage, female percentage and intersex percentage for plants, solvents and concentrations.

Hypothesis Test Summary	
Null Hypothesis	Significance
The medians of survival percentage are the same across categories of plant	0.813
The medians of female percentage are the same across categories of plant	0.637
The medians of intersex percentage are the same across categories of plant	0.097
The medians of survival percentage are the same across categories of solvent	0.145
The medians of female percentage are the same across categories of solvent	0.000
The medians of intersex percentage are the same across categories of solvent	0.007
The medians of survival percentage are the same across categories of concentration	0.125
The medians of female percentage are the same across categories of concentration	0.092
The medians of intersex percentage are the same across categories of concentration	0.175

Note: Asymptotic significances are displayed. The significance level is 0.05.

Table 3. Qualitative analysis of phytochemicals in different solvent extracts of *Mucuna pruriens* seeds and *Asparagus racemosus* Roots.

Plant	Solvent	Phytochemical Groups						
		Tannin	Saponin	Alkaloid	Carbohydrate	Glycoside	Flavonoid	Steroid/ Terpenoid
Mucuna pruriens Seeds	Aqueous	+	+	-	-	-	++	+
	Ethanol	+	+	-	-	+	+	+
	Methanol	++	-	+	-	++	+	+
	Successive	-	-	+	+	-	+	+
Asparagus racemosus Roots	Aqueous	+	+	-	-	-	+	+
	Ethanol	+	-	-	+	+	++	+
	Methanol	+++	-	-	++	++	+	+
	Successive	-	-	+	++	-	+	+

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Table 4. Antiradical activity, total phenol content and total flavonoid content in different solvent extracts of *Mucuna pruriens* seeds and *Asparagus racemosus* Roots.

Plant	Solvent for Extraction	Antiradical activity (%)	Phenol Content (mg of GAE/g dry weight)	Flavonoid Content (mg of RE/g dry weight)
Mucuna pruriens Seeds	Aqueous	0.298	658	11.72
	Methanol	18.265	578	0.233
	Ethanol	4.404	557	3.71
	Successive	1.93	271	1.99
Asparagus racemosus Roots	Aqueous	2.601	501	6.56
	Methanol	18.265	653	7.7
	Ethanol	98.522	653	22.08
	Successive	0.028	629	9.12

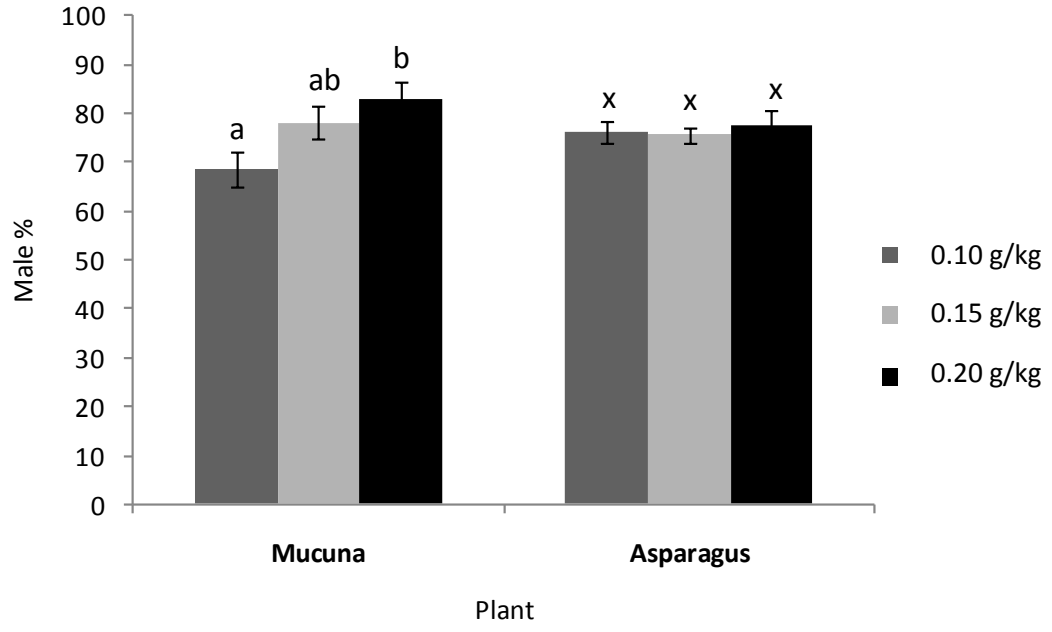

Figure 1. Percentage of males in tilapia fed diets containing different concentrations of *M. pruriens* seeds and *A. racemosus* roots extracts. Different alphabets above column indicates significant difference (P<0.05) in means.

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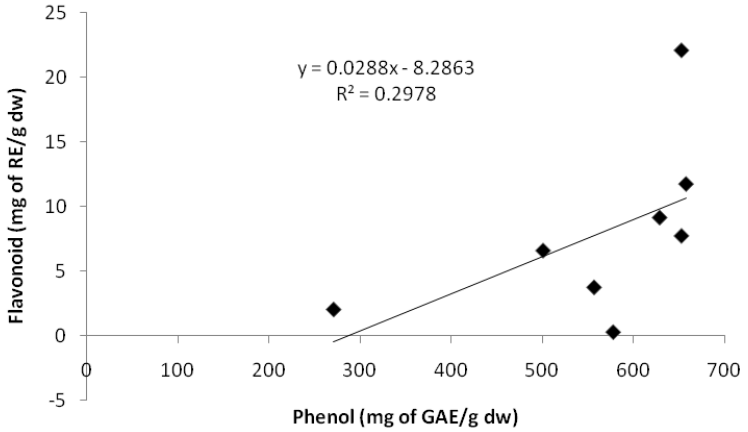

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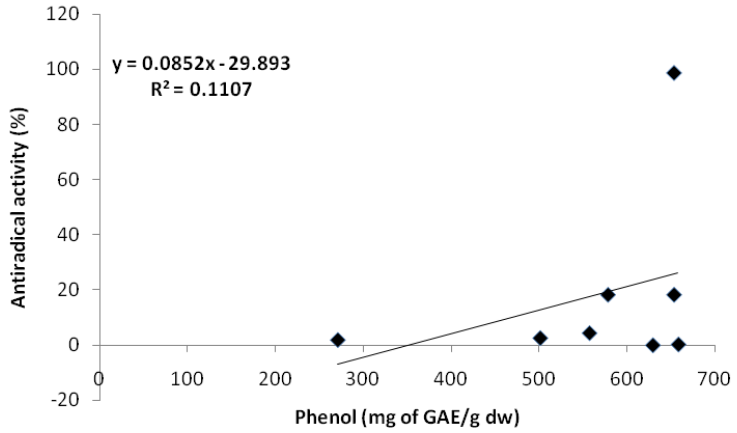

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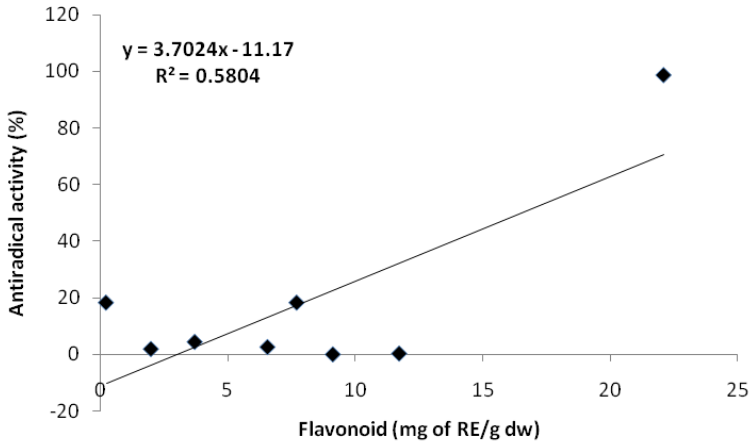

c)

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Figure 2. Correlation between total phenolic content and total flavonoid content (a), total phenolic content and antiradical activity (b) and total flavonoid content and antiradical activity (c) of different solvent extracts of *Mucuna pruriens* seeds and *Asparagus racemosus* Roots.