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Effect of different plant extracts on microbial population in the frozen African Catfish (*Clarias gariepinus*) semen

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ABSTRACT

Fish sperm cryopreservation has been attempted on roughly freshwater and marine species since 1953. This study sought to assess the potential of various plant extracts to function as natural antimicrobial agents in the frozen semen of African catfish (*Clarias gariepinus*). Diluted sperm was packaged in 0.25ml straws and left for 10min equilibration at 4°C. Following equilibration, the straws were exposed to liquid nitrogen vapor for 10 min and plunged into the liquid nitrogen (-196°C) and then thawed in a water bath at 35°C for 20s. Sperm samples were put into sterile 1.5 ml tubes immediately after thawing and the microbial count was detected with classical microbiological culture method. In the results of microbiological analyses, these tree plant extracts especially *Echinacea purpurea* were found highly effective for decreasing bacterial contamination levels of African catfish (*C. gariepinus*) semen. These plant extracts may have the potential for antibacterial effect, and they can be useful for the dilution of semen.

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Introduction

African catfish (*C. gariepinus*) is an economically important freshwater fish species, with a growing demand for its consumption both locally and internationally. It is one of the most important benthopelagic fish species cultured in South East Asia, Africa and also in Europe (1). It has also been considered as one of the most suitable species for aquaculture.

In recent years, there has been a growing interest in the use of natural plant extracts as an alternative to synthetic antimicrobial agents due to their safety, efficacy, and sustainability. The use of herbal extracts for various health benefits has been gaining interest in recent years, not only for human but also for animals.

Many plants have been found to possess antimicrobial properties that can be used to control microbial growth and improve the quality with ultimately enhancing the productivity and sustainability of aquaculture. The use of natural plant extracts with antimicrobial properties may offer a safe and effective alternative to synthetic antimicrobial agents, which can have negative effects on the environment and human health (2).

Plants have various natural compounds which can be extracted by different methods. One of these methods is steam distillation. In steam distillation, fresh herbs are placed in the retort and exposed to hot steam. With the heat of the steam, the oil sacs in the plant explode and the essential oil is mixed with the steam then this mixture enters the cooling channel. In the cooling channel, it is condensed with cold water and accumulated in the oil storage tank. After cooling, the water and oil mixture are separated. The remaining oil is removed and collected for further usage. The study of plants and their derived compounds as potential therapeutic agents is the primary focus of herbal pharmacognosy (3). Previous studies have demonstrated that adding different antioxidants to semen extenders can enhance the maintenance of sperm quality during the freeze-thawing process, in comparison to the control. However, there is a scarcity of literature examining the impact of medicinal plant extracts as a source of antioxidants on sperm quality (4-7).

Microbial contamination is a major challenge in the preservation of fish semen and can negatively impact its quality. Therefore, there is a need to develop effective methods for preserving fish semen, which can involve the use of natural plant extracts with antimicrobial properties. The availability and quality of gametes throughout the year is an important factor to ensure a continuous supply of fish (8). The quality of fish semen is a critical factor in the successful reproduction and production of this species in aquaculture.

The aim of this study was to investigate the effect of different plant extracts on the microbial population in cryopreserved African catfish (*C. gariepinus*) semen, with the potential to improve its quality and enhance the success of artificial reproduction techniques in aquaculture.

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Materials and Methods

Plant extracts

Plant extracts of Rosemary (*Rosmarinus officinalis*), Echinacea (*Echinacea purpurea*) and St. John's Wort (*Herba hyperici*) were purchased from a local producer company in Antakya/Hatay, of which extract was obtained by steam distillation, and approved by nutritionist the Faculty of Veterinary Medicine of Trakia University.

In steam distillation, fresh or dried herbs are placed in the retort and exposed to hot steam of water. With the heat of the steam, the oil sacs in the plant explode and the essential oil was mixed with the steam than this mixture enters the cooling channel. In the cooling channel, it is condensed with cold water and accumulated in the oil storage tank. After cooling, the water and oil mixture are separated. The remaining oil is removed and collected for further usage.

Brood stock

Mature wild African catfishes were captured from Golbasi Lake in Hatay (Turkey) in May by the fisherman's for the fish market. Sexually mature broodfishes were kept and separated in tanks supplied with continuously (2.5L min-1) well-aerated water (Temperature was 26ºC and dissolved oxygen was 10 mg L-1 which was measeured with AZ 86031 Combo Water Meter) for gender determination and for transporting to the fish markets (Figure 1).

Removal of testicles and preparation of semen straws

The brood stock fish immobilize by pithing with a pointed knife just behind the operculum before dissection and testicles were removed from five male catfish and perforated with a needle. The testicles were placed on sterile petri dishes (Figure 2). Sperm was gently squeezed out and collected in dry tubes.

The collected ejaculate was pooled and split into four equal aliquots and diluted with based NaCl, KCl, $CaCl_2$, Tris extender (8) containing plant extracts (0.02% Rosemary (*Rosmarinus officinalis*) Echinacea (*Echinacea purpurea*) (0.5 mg/mL) and St. John's wort (*Herba hyperici*) (0.06 mg/mL). The final concentration of motil spermatozoa per straw was approximately $2x10^8$, which was counted with Neubauer Hemocytometer (9).

Figure 1. Male African catfish (*Clarias gariepinus*).

Figure 2. Testicles of male catfish.

Cryopreservation of semen

After equilibration, the semen samples (straws) were placed 4 cm above the liquid nitrogen surface in the box and were frozen in liquid nitrogen vapour (-140°C) for 10 min. The straws were kept in liquid nitrogen (-196°C) in a container until thawing. To thaw, the straws were removed from the liquid nitrogen and submerged in a water bath at 35°C for 20s. Semen samples put into sterile 1,5 ml tubes immediately after thawing and the microbial process was put into implementation.

Bacterial count

The microbial population was measured by counting the number of colony-forming units (CFUs) of bacteria present in the semen samples before and after treatment with the plant extracts. For bacteria count by classical microbiological analyses, 10µl from fresh diluted semen and after thawing semen containing plant extract was taken and streaked into three sets of blood agar (containing 7% sheep blood). Each set of blood agar was incubated +4°C, +22°C and +37°C aerobically, for 24-72 hours. After the incubation colony counts were calculated and determined the bacteriological quality of samples (10).

Statistical analysis

The statistical analysis of the obtained data was performed by the General Linear Model (GLM). All of the values were reported as the means and standard error of the mean (means ± SEM). The level of significance was accepted if the p-vale was less than 0.05.

Results

In the results of microbiological analyses, the number of the aerobic bacterial count was detected in control $80x10^2$ at +4°C, $96x10^2$ at +22°C and very $100x10^2$ at +37°C. And the bacterial count for plant extract added semen, no bacterial growth +4°C, $7x10^2$ at +22°C and $3x10^2$ +37°C thawed semen containing Rosemary (*Rosmarinus officinalis*, 0.02%), no bacterial growth +4°C, $1x10^2$ at +22°C and no bacterial growth +37°C thawed semen containing Echinacea (*Echinacea purpurea*, 0.5 mg/mL), and no bacterial growth +4°C, $2x10^2$ at +22°C and $6x10^2$ +37°C thawed semen containing St. John's wort (*Herba hyperici*, 0.06 mg/mL) (Table 1 and Figure 3).

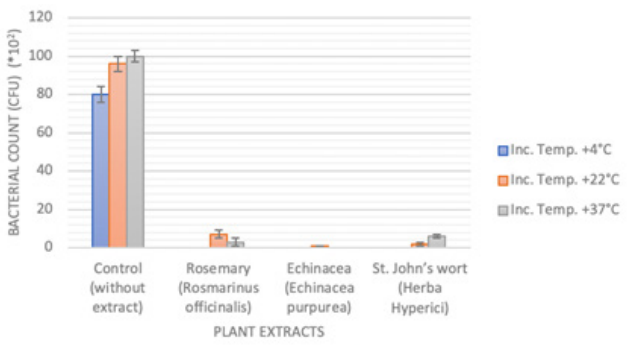

Figure 3. Results of effects of plant extracts (Mean±Stdev).

Table 1. Results of effects of plant extracts in CFU (mean±SEM).

Plant extracts	Incubation Temp.		
	+4°C	+22°C	+37°C
Control (without extract)	80±2.30 x$10^{2\ A,b*}$	96±2.30 x$10^{2\ B,c}$	100±1.73 x$10^{2\ B,c}$
Rosemary (*Rosmarinus officinalis*)	$0^{\ Aa}$	7±1.15 x$10^{2\ B,b}$	3±1.15 x$10^{2\ AB,ab}$
Echinacea (*Echinacea purpurea*)	$0^{\ Aa}$	1±0,00 x$10^{2\ B,a}$	$0^{\ A,a}$
St. John's wort (*Herba hyperici*)	$0^{\ Aa}$	2±0.57 x$10^{2\ B,ab}$	6±0.57 x$10^{2\ C,b}$

*Different superscript capital letters in the same line and small letters in the same column indicate statistical significance (P<0.05).

Discussion

In animal husbandry, several direct and indirect approaches to improve the reproduction process, starting from feed supplementation/additives, through drugs/hormonal application, until the control of reproductive media (11).

In recent years, the effects of additives have played an important role also in the research focusing on reproduction in the aquaculture sector. In fish, some studies showed that valuable compounds from plants may be used in the form of extract in the feed (12). Especially, in African catfish, the importance of plant extracts on enhancing fertility and reproductive performance has been highlighted by several authors. Francis et al., (13) reported that diets supplemented with *Vernonia amygdalina* increased fertility in African catfish (*Heterobranchus bidorsalis*). In the similar studies, with feed enriched of local African medical plants *Kigelia africana*, *Sesamum indicum* and *Croton zambesicus* researchers reported enhanced reproductive performance as fertilization, hatching, percentage of number of sperm cell, sperm motility, sperm survival time, percentage of active sperm (14, 15). These findings are contradicting contrast to those of Ekanem et al., (16) who reported negative effects on the fecundity, and gonad development of *C. gariepinus* when fed to dietary inclusion of native African plants; *Stachytarpheta jamaicensis* and *Garcinia kola*. Meanwhile, this is consistent with a previous report of Owoyemi, et al., (17) who experimented with the gel extract of Aloe vera on male African catfish sperm, and found adverse effects on sperm parameters especially, sperm motility, live ability concentration and morphology. All these results show unequivocally that firstly, an extract must be able to be digested and absorbed in the fish and secondly, the lack of saponins and flavonoids, which are accepted as the main bioactive compounds in phytoextracts, causes sex inversion and fertility impairment (18). In addition to plant extract effects, other scientific interests are to find alternatives to antibiotics to control bacteria that contaminate the ejaculate (19). Antibacterial effects of plant extracts and using them to decrease bacterial contamination in semen were reported in different animals. Extracts from 45 plant species, belonging to 28 families, are reported to control sperm function in several animal species (3). Ngu, et al, (20) tested extracts of *Careca papaya*, *Vernonia amygdalina* and *Jatropha curcas* against bacteria in buck (male goat) semen and they reported that these extracts showed appreciable inhibition activity on the isolates, therefore, be used in the control of microorganisms in the ejaculate. Mazurova et al., (21) used natural substances gallic acid, methyl gallate, ethyl gallate, propyl gallate, octyl gallate, thymol, carvacrol and eugenol for the decontamination of boar semen. The authors suggested that these substances successfully may be used for boar semen decontamination and with lower toxicity compared to other substances. Unfortunately, to the best of our knowledge, no information was found in the scientific literature on the decontamination of semen in African catfish. The results of microbiological analyses of the present study exhibited that all tested plant extracts were effective for decreasing bacterial contamination level of African catfish (*C. gariepinus*) semen. The *Echinacea purpurea* was found to have the highest inhibition activity. The antimicrobial effect of Echinacea purpurea is attributed to the chemical composition and in particular to the presence of phenolic compounds and cichoric acid but it definitely will not be effective in dissolving biofilms formed by pathogenic bacteria (22). Meanwhile, it is well known that the antimicrobial properties in *Rosmarinus officinalis* per sraw also are mainly due to phenolic compounds and rosmarinic acid. Numerous researchers have been carried out to study the potential antimicrobial activity of rosemary extracts on frozen semen in several species (23-26) but no one experiment was reported in fish. Despite the good antimicrobial activity, the presence of flavonoids in *Herba hyperici*, which is detected using thin-layer chromatography, predetermines the limited application in fish due to the possibility of sex inversion and fertility impairment.

Sperm cryopreservation is a common practice in aquaculture, there for application of good sanitary control is the key moment regarding the quality of cryopreserved fish sperm. The tested plant extracts expressed inhibition activity and have the potential to could be a natural alternative for the prevention and control of fish semen.

In the results of microbiological analyses of this study, these tree plant extracts especially *Echinacea purpurea* were found highly effective for decreasing bacterial contamination levels of African catfish (*C. gariepinus*) semen. It is considered in future studies to investigate sperm motility and the effect of plant extract on sperm cells. For now, these plant extracts may have the potential for an antibacterial effect and they can be useful for dilution of fish semen.

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No authors have a conflict of interest relative to this work.

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Authors' contribution

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