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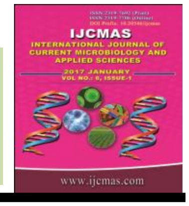

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Evaluation of *Pyrus pashia* Leaf Extract as a Disinfectant for Rainbow Trout (*Oncorhynchus mykiss*) Fertilised Eggs

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A B S T R A C T

In hilly areas, local population with their traditional knowledge utilize several medicinal herbs and plants parts for treating injuries and aliments of their livestock, agricultural crop and human population. Among these *Pyrus pashia*, commonly known as Indian pear or Mehal is hardy wild mid hill fruiting tree. Crude leave extract traditionally used for treating infection of toe finger particularly during rainy season. Fruit juice and ripe fruits of *P. pashia* used for treating eye injury and mouth sours. With a view to minimize use of chemical disinfectant in hatchery and to find out a locally available suitable herbal substitute for disinfecting trout eggs, present comparative study was undertaken to observe the effect of leaves extract of *Pyrus pashia* on egg survival and hatching during incubation phase.

Keywords

Rainbow trout, fertilised egg loss, hatchery incubation, disinfectant, leaf extract, *Pyrus pashia*

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Introduction

Among cultivable coldwater fishes, rainbow trout, *Oncorhynchus mykiss* contributes substantially in upland aquaculture production in India (Vass 2012). The species is widely preferred for farming owing to its hardy nature, tolerance to wide temperature fluctuations and simple breeding protocol (FAO 2012). Fertilised egg of rainbow trout takes a long incubation period of 3-14 weeks for hatching under varied thermal regimes (Woynarovich *et al.*, 2011). Factors like lower sperm motility, over- ripening / untimely egg tripping, poor egg quality, physical injury handling and microbial infection greatly influence fertilisation and survival of eggs during hatchery incubation. Exposure of trout eggs to direct light or higher illumination for longer period during incubation, fungal infections and frequent disturbance of eggs in rearing troughs may also reduce survival of eggs and fry (Bell *et al.*, 1971; Barker *et al.*, 1989; Griffiths 1991; Shepherd and Bromage 1992). Environmental parameters like temperature, pH, dissolved Oxygen, ammonia etc. also plays important role in spread of infections and survival of eggs. Bacteria to some extent are responsible for salmon egg loss under a complex interaction between eggs, microbes and water quality parameters (Shepherd and Bromage 1992). During various developmental stages of eggs in hatchery, under unhygienic conditions, opportunistic and pathogenic microbes inhabit over the eggs surfaces, thus, chances of egg damage increases many folds (Barker *et al.*,1991). Although, higher survival of trout eggs in hatchery could be achieved by selection of healthy brooders, maintaining optimum water quality parameters (Woynarovich *et al.*, 2011). However, during egg incubation phase, heavy loss of eggs has been reported due to microbial infections (Anon 2013). With growing rainbow trout farming in mid hill areas, demand for rainbow trout fingerlings is also rising day by day. To meet increasing seed demand, more numbers of eggs are incubated in unit area, thus chances of infection also increases several times (Jeff *et al.*, 2005). Improving water quality supply of hatchery and maintaining cleanness in egg rearing troughs through regular removal of dead and decayed eggs, shells and other substrates help in reducing egg loss. However, in commercial trout seed production units, practically it is not always possible to maintain all the desired parameters under optimum range. To maintain optimum hygienic conditions, various anti fungal and antibacterial chemicals have been tested in trout hatcheries with varying degree of protection. This includes malachite green, formaldehyde, iodine, hydrogen peroxide, bronpol and sodium chloride (Wanger *et al.*, 2008). Malachite green was earlier considered as one of the strong antifungal agent for treating fish eggs. However, malachite green was banned worldwide in year 2002 owing of its harmful effects on animal health (Van West 2006). Exposure of incoming hatchery water, by U.V radiation significantly reduce trout egg loss in hatchery (Heikkinen *et al.*, 2013). At the same time, studies has also revealed that ozone (Forneris *et al.*, 2003), H_2O_2 (Gaikowski *et al.*, 1998) and UV radiation (Heikkinen *et al.*, 2013) may decrease egg hatching percentage during trout eggs incubation. To achieve better egg survival, antibiotics are also used. Unregulated use of chemicals and antibiotics in fish hatcheries and farms elicit harmful effects on fish and aquatic environment. Negative effect of several chemicals and antibiotics used in hatcheries are well known. With increasing awareness towards these effects, safe methods are being explored to minimize such losses and their side effects (Srinivasan *et al.*, 2001). Many herbal extracts or paste for curing health disorders are now widely being preferred and have been tried in treating fish health disorders (Madhuri *et al.*, 2012a; Pandey *et al.*, 2012b; Dey and Chandra 1994, 1995; Ramasamy *et al.*, 2011). In hilly areas, local population with their traditional knowledge utilize several medicinal herbs and plants parts for treating injuries and aliments of their live stock, agricultural crop and human population (Negi *et al.*, 2011). These plants contain active ingredients which act as antimicrobial and antifungal agents. Among these *Pyrus pashia* (Buch-Ham ex D.Don), commonly known as Indian pear or Mehal is hardy wild mid hill fruiting tree belongs to family Rosaceae and found in the mid Himalayan areas. The tree generally grows on sandy loamy soil at a temperature range from -10 to 35 ^{0}C. The bark leafs and fruits are traditionally used both for consumption and also as medicine (Jambey *et al.*, 2012). Strong antioxidant activity with free radical scavenging activity has also been reported in flower extract of *P. Pashia* (Jianmin Hea, 2015). Crude leave extract traditionally used for treating infection of toe finger particularly during rainy season. Fruit juice and ripe fruits of *P. pashia* used for treating eye injury and mouth sours (Negi *et al.*, 2011). With a view to minimize use of chemical disinfectant in hatchery and to find out a locally available suitable herbal substitute for disinfecting trout eggs, present comparative study was undertaken to observe the effect of leaves extract of *P. pashia* on egg survival and hatching during incubation phase.

Materials and Methods

Plant material: *P. pashia* is an angiosperm commonly known as Mehal or Mol in Hindi. The tree is widely distributed in mid Himalayan areas in between 750-2600 msl and generally grows to a height of 3-6 m in sandy loamy soil. Although, small pear shaped fruits are edible but, they do not fetch any market price and mostly consumed by wild animals. Locally crushed green leaves extract is used traditionally by hill people for curing infections of toes.

Preparation of disinfectants: Newly budding reddish green leaves of P. pashia were collected from the trees located in fish farm of ICAR-DCFR Field Centre, Champawat in Uttarakhand state of India, located at an altitude of 1620 msl. Collected leaves were washed thoroughly in tape water and then allowed to dry under shade for 10 days. After complete drying, leaves were grinded and 200 g fine powder was made. The powder was soaked in 800 ml 100% ethanol and placed in a shaking incubator for 24 hours. Soaked leaves powder in the above solvent was filtered through 20µ mesh size filter paper. Filtered extract then placed on rotary evaporator and evaporated till volume reduced to 200 ml. The solution stored in fridge in air tight bottles at 4^0C temperature. Before using the extract for egg disinfection, a 100 ml solution of leave extract was diluted with 900 mL of distilled water to make it 10% and further concentration made accordingly (Lin *et al.*, 1999). *Pyrus pashia* was prepared from naturally dried fallen leaves and above methodology was followed for preparing extract of the dry leaves. Stock solution of iodine named Betadine (Povidone iodine) containing 0.5% w/v iodine (Win Medicare) was procured from the local market and diluted to a concentration of 100mg/l.

Rainbow Trout Egg Striping: Mature healthy rainbow trout brooders with mean weight/length of 679.90±11.97g/371.00 ±11.10mm were randomly collected from brooder raceways and brought to hatchery for stripping. Approximately 35,000 nos. of eggs were realized from 23 nos. of female rainbow trout brooders. The milt was poured over the eggs and kept for 15 minutes in dark conditions to accomplish adequate fertilization. All batches of fertilized eggs were mixed together to get uniformity of egg stock. For different treatment, initially fertilized eggs were equally divided in four trays.

Disinfection of trout eggs: Following standard methods of egg disinfection, fertilized eggs were exposed in three treatments (Anon 2009). Before adding water in fertilized eggs, egg of first tray/trough were exposed in 10% green leaves extract (GL) for 15 minutes, washed thoroughly in hatchery water and equally divided in three rearing troughs @ 2500±250/trough for further incubation. Fertilized eggs of second tray were similarly exposed in 10% prepared dry leaves extract (DL) for 15 minutes, washed and equally placed in three troughs @ 2500±250/trough. Eggs of third troughs dipped in 100mg/L Iodine solution (I) for 15 minutes, washed and placed in three troughs @ 2500±250/trough for incubation. The dose in GL, DL was arrived through earlier observations made in hatchery under similar conditions. Eggs of fourth trough were thoroughly washed in tape water and transferred to incubating troughs measuring

45x45x18 cm in size in replicates and kept as control without any exposure. These egg rearing troughs were placed over horizontal rectangle FRP tub (225x50x21cm size) with a holding capacity of four egg troughs/tub. Normal water flow rate of 6-8 L/min was maintained during egg incubation period. Daily egg damage in each trough was recorded for 42 days (6th week) till hatching of the eggs. Uniformity in hatching of egg also recorded.

Physicochemical Parameters: Water quality parameters of experimental troughs were analyzed at fortnightly intervals while water temperature recorded every day to ascertain variation of limiting important parameters. Water parameters were analyzed by WTO15 Multi-water Parameters Testing KIT-HI Media and Dissolved Oxygen by Winkler method. Dead and decomposed eggs were routinely taken out from all the 12 egg incubation troughs and counted. At fortnightly intervals, rectangle egg rearing troughs were cleaned without disturbing eggs to maintain optimum rearing conditions for the developing eggs.

Statistical analysis: Mean value of parameter were subjected to one-way analysis of variance (ANOVA) to study the treatment effect and Duncan's Multiple Range Tests (DMRT) were used to determine the significant differences between the mean value. Comparisons were made at 5% probability level. All the data were analyzed using statistical package SPSS (Version 16) (SPSS Inc., Chicago, IL, USA).

Results and Discussion

In three exposures and control, numbers of weekly egg damage in each rearing troughs was studied. Mean egg damage in green leaves extract (GL) was significantly lowest ($P<0.05$) in I, II and VI week of incubation however, in III week, no difference in egg loss was recorded in all three exposures and in control troughs. Except II, III and V week, Iodine exposed eggs showed significantly higher egg loss. DL exposed egg showed significantly lower egg loss only in II week. Mean percent egg loss under three exposures and control is presented in Table-1. Total mean percent egg loss is given in Fig-1. Physicochemical parameters (Table-2) remained under normal limits. However, water temperature of hatchery varied between 5.0-11.5 ^{0}C (Fig-2). Highest mean ±SE hatching percent of 62.66±4.33 observed in GL treated eggs followed by 42.00±5.80 in C, 35.33 ±3.17 in DL and 15.66± 4.04 in I (Table 3).

The major objective of this study was to find out suitability of locally available plants leaves extract for disinfecting trout eggs. Among various plants, *Pyrus pashia* is widely found in mid Himalayan areas and grows in sandy loamy soil. During rainy season, mid hill local people commonly uses crumpled green leaves extract of *P. pashia* for healing of Athlete's foot disease which is assumed to be of a mixed fungal and bacterial etiology having high resistance against common antibacterial and antifungal medicines. Fertilized eggs of rainbow trout (*Oncorhynhus mykiss)* under water temperature range of 3.9-14.4°C takes a long period from 21-100 days (Woynarovich *et al.*, 2011). During this prolonged incubation period, fertilized eggs constantly come in contact of diverse environmental and microbial organism. With elongation of incubation period, chances of egg damage increases many folds due to growth and colonization of bacteria and fungus over the eggs in rearing facilities. A number of occasions, higher load of microbes also get entry through stream water which is normally maintained @t 6-8 L/minute (7200-10080 L/day) in rearing troughs. In flow- through hatcheries system, where retention of incoming water is for a shorter duration, effective control of microbial infections is a major problem. Large egg scale loss with wide fluctuations in environmental parameters in a rainbow trout hatchery has been reported (Anon 2013). Several external disinfectants like malachite green, iodine, hydrogen peroxide, bronopol, salt, formalin, UV radiation and herbal extracts have been tried to control such infection with varying degree of success. (Kimura *et al.,* 1976; Mitchill and Collins, 1997; Wanger *et al.*, 2008; Jambey *et al.,* 2012). Under present observation, compared to non treated control group (C) where egg damage was 12.81%, egg damage in dry leaves extract (DL) and iodine (I) was significantly higher and was 15.28% and 23.90% respectively. However, green leaves extract (GL) showed significantly ($p < 0.05$) lower total percent egg mortality of 4.61% till 6th week of incubation indicating probable minimization of external infections in eggs. The green leaves extract (GL) of *P.pashia* helped in reduction of egg loss in initial weeks when likelihood of infection spread is more thus, gave higher survival rate. This may primarily be attributed to presence of phenolics alkaloids, and saponins compounds in leaf extract of *P. pashia* which might be helping in inhibition of microbial infection particularly in initial weeks of incubation. Through disc diffusion test, antibacterial activity of *P. pashia* against *Klebsiella pneumonia*, *Shigella flexneri* and *Escherichia coli* has been demonstrated (Saklani and Chandra 2012). Antioxidant activity and presence of phenolic compounds in edible flowers of *P. pashia* has also been evaluated by researchers (Hea *et al.,* 2015). Saponins compound found in many herbs act as antibacterial agent and herb, *Sorghum Bicolor* containing saponins compounds exhibits antibacterial activity against gram+ bacteria (Soetan *et al.,* 2006). Same may be true in case of *P. pashia* extract. Apparently, normal development in GL exposed eggs in rearing troughs observed. Iodine (I) treated eggs showed highest percent egg loss in present study followed by DL treated eggs (Table 1). Dry leave extract (DL) of *P. pashia* was prepared by picking dropped old leaves which got exposed to direct sunlight and rain and then extract was prepared. The DL did not showed higher survival compared to control ones probably due to prior exposure of leaves in rain and sun resulting to leaching of beneficial bactericidal compounds like saponins and other alkaloids (Fig1). Microbial killing takes place either due to oxidation or halogenations of microorganisms (Bandrick *et al.,* 1967) in iodine treated fertilized eggs. Groups of I treated fertilized eggs showed maximum egg mortality during incubation period. Iodophors are considered as a reliable disinfects in egg treatment in hatchery (Wade and Michael, 1967) and adequate dose and exposure period gives better survival in rainbow trout eggs (Fowler and Banks, 1991). Un-rinsed fertilized eggs were used under present study for exposing fertilized eggs in iodine solution. Lower retention of iodine in unrinsed fertilized eggs with lower dose might be the probable reason for higher egg loss. Similar to our observation immediate decline of iodine in unrinsed eggs has been reported in earlier study conducted by Chapman and Rogers 1992. Although, chances of iodine toxicity may not be the possible reason for egg mortality in present observation as 100mg L^{-1} dose of iodine is not harmful for rainbow trout eggs (Amend 1974; Wanger *et al.*, 2008). However, study of Wright and Snow 1975, demonstrated that lower dose of iodine was not able to inhibit *Aeromonas liquefacience* growth over the eggs and higher dose of 200mgL^{-1} only could control the infection. In hatchery troughs, fertilised eggs which encounter frequent water temperature variations due to lower water volume appear to make them more sensitive under intensive rearing. Usually 35-45 days are taken at water temperature range of 5-11.5 ^{0}C for hatching at Champawat hatchery in flow through system, however damaged un-removed eggs sometime act as a substrate for bacterial and fungal growth. It was found that with progression of hatching period, accumulation of organic residues below the troughs bottom resulted in rapid multiplication of organisms and thus in this process, higher egg loss has been observed.

Table.1 Mean percent ±SE trout egg loss under three exposures and control. Mean values bearing different superscript in a column differ significantly (P<0.05)

S. No.	Treatment	Mean % SE egg loss during different weeks of incubation					
	Week	I	II	III	IV	V	VI
1.	Green leaves Extract (GL)	0.34^{c} ±0.05	$O.53^{d}$ ±0.08	0.75^{a} ±0.08	0.67^{c} ±0.14	0.88^{b} ±0.10	1.44^{c} ±0.13
2.	Dry Leaves Extract (DL)	3.07^{ab} ±0.15	1.12^{c} ±0.02	5.16^{a} ±2.12	1.11^{bc} ±0.17	1.68ab ±0.01	3.14^{b} ±0.09
3.	Iodine (I)	4.24^{a} ±0.68	1.75^{b} ±0.13	6.40^{a} ±2.76	1.41^{ab} ±0.04	3.4^{a} ±1.40	6.70^{a} ±0.30
4.	Control(C)	2.40^{b} ±0.17	2.36^{a} ±0.31	1.20^{a} ±0.14	1.65^{a} ±0.22	1.15^{ab} ±0.074	4.05^{b} ±0.47

Table.2 Weekly physicochemical parameters range during hatchery incubation of eggs

Parameter	I	II	III	IV	V	VI
Air temp Min/Max ^{0}C	8.0-11.0	8.5-11.3	6.5-14.5	8.0-14.5	10.0-16.0	16.0-18.0
Water Temp Min/Max. ^{0}C	5.4-7.2	7.5-8.5	7.0-8.0	8.0-9.1	8.8-10.6	11.0-11.5
pH	7.0-7.8	7.0-7.2	7.0-7.3	7.0-7.2	7.1-7.2	7.0-7.2
DO(ppm)	7.5-8.0	7.5-8.0	6.5-7.2	7.5-8.0	7.0-8.5	6.6-7.2
Hardness(ppm)	25-30	25-30	25-30	25-30	25-30	25-30
Iron(ppm)	0.1-0.2	0.2-0.3	0.2-0.3-	0.1-0.2	0.1-0.2	0.2-0.3
Nitrate(ppm)	0.1-0.2	0.1-0.2	0.1-0.2	0.2-0.3	0.2-0.3	0.1-0.2
Chloride (ppm)	30-40	30-40	30-40	30-40	20-30	20-30

Table.3 Percent egg hatching on first day. Mean values bearing different superscript in a column differ significantly (P<0.05)

S. No.	Treatment	Mean % egg hatching on first day	SD
1.	Green leaves Extract (GL)	62.66^{a}	±4.33
2.	Dry Leaves Extract (DL)	35.33^{b}	±3.17
3.	Iodine (I)	15.66^{c}	±4.04
4.	Control(C)	42.00^{a}	±5.80

Fig.1 Total mean percent egg loss under three exposures and control. Bars with different superscripts differ significantly (P < 0.05)

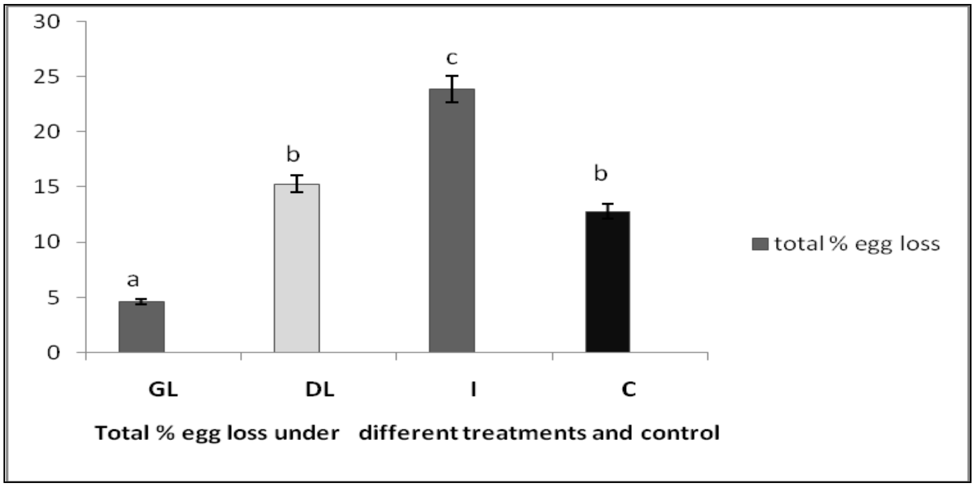
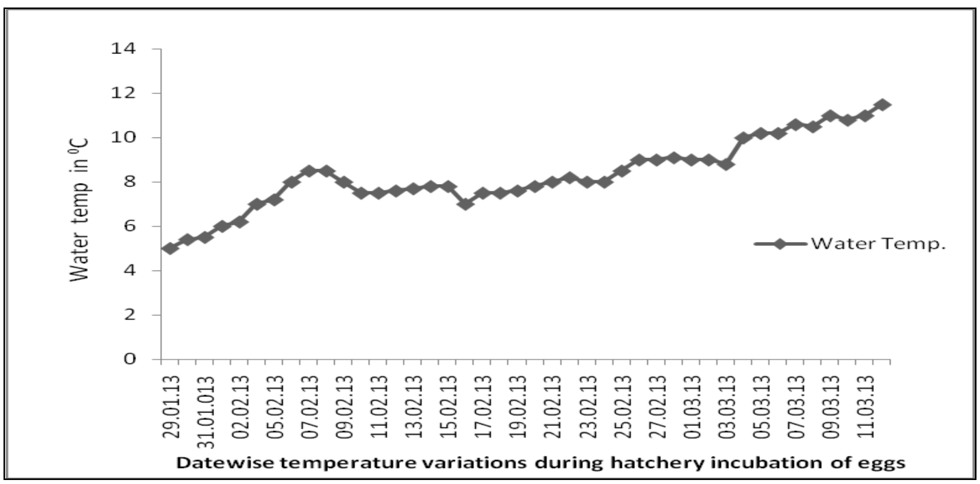


Fig.2 Water temperature variation in egg incubation trays during incubation period.



Beneficial effects of given disinfectant may get diluted with elongation of incubation period. Reports have indicated that few bacterial isolates may be inhibited at a lower iodine dose of 50mg L^{-1} and 30mg L^{-1} (Cipriano *et al.*, 2001) Water temperature during study period varied between 5-11.5 ^{0}C (Fig 2), which was well under acceptable limit and probably may not have elicited any adverse influence on egg development. According to Huet 1970, water temperature tolerance limit of rainbow trout eggs and embryo is 5-15 ^{0}C with optimum level of 8-14 ^{0}C. Among other parameters, hardness value may also affect the normal developmental stages (Molony 2001), however, hardness was at lower side in between 20-30 ppm and to some extant might have affected the survival of eggs in present study. Though, acceptable normal pH level is reported in between 6.5-8.0 (Huet 1970; Molony 2001), under present study pH was optimum and varied between 7.0-7.8 during the entire period of egg rearing while chlorides nitrate and iron values were found under tolerable limit. At the time of hatching, uniformity in release of yolk sac larvae from the eggs and shorter time durations may also be considered a sign of well being. Highest mean ±SE hatching percent of 62.66±4.33 with lower mortality in 6th week was observed in GL treated eggs followed by 42.00±5.80 in C, 35.33±3.17 in

DL and 15.66±4.04 in iodine solution demonstrating trend similar to egg loss in above exposures presenting superiority of GL over other treatments in regards to hatching (Table 3). Literature referred has revealed that so far no studies has been made on the use of *P. pashia* leaf extract as rainbow trout egg disinfectant and this may probably be the first report in regards to use of *P. pashia* as trout egg disinfect. The plant is widely available in mid hill areas and leaf, fruits and flowers are being mainly used by wild animals. In mid hill areas, farming and seed production of rainbow trout is commonly practiced by trout grower, use of locally available suitable plant extract may save money as well as use of harmful disinfects.

In conclusion, the results of present study suggest that freshly procured shade dried green leave extract of Pyrus pashia @ 10% could be used as egg disinfectant in rainbow trout eggs without any harmful effects on hatching under normal physicochemical parameters of hatchery water and significant higher egg survival was obtained in present study. Further detailed studies may add for use of this plant extract.

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