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Evaluation of anaesthetizing efficacy of clove oil in *Channa punctatus*

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Abstract
In the present work, the efficacy of anaesthetising agent viz. Clove oil on *Channa punctatus* has been investigated. Experimental protocol was based on analysis of induction and recovery time with different concentrations of above anaesthetic to determine their optimum concentration. Histological studies were carried out to assess the stress induced changes caused by the application of different concentrations of clove oil. Well acclimatized fishes, divided into three groups, were exposed to three different concentrations (50, 100, 200µl/L) of anaesthetic and the changes in the above parameters were recorded. Based on the results, it is concluded that clove oil at concentration of 100µl/L is the most suitable anaesthetizing concentration due to its quick induction of anaesthetization and longer recovery time with the least stress as evident from the histological architecture of gills and buccal epithelium of *C. punctatus*.

Keywords: clove oil, *Channa punctatus*, anaesthetization, gill, buccal epithelium

1. Introduction
In Aquaulture, the manipulative processes like capturing, handling, transportation of fish cause the alterations in fish physiology and behaviour, which can be reduced by application of various available anaesthetics [1, 2]. Hence, use of anaesthesia is an important aspect in aquaculture, wild fish collection and fisheries management. During the surgical procedures of fish in the laboratories, anaesthetics are also required to immobilize the fish to prevent integumentary damages, associated osmoregulatory disturbances, and other physiological changes [3, 4, 5, 6]. Choice of an effective anaesthetic agent is based on some important factors like efficacy, cost, availability, ease of use, fish species, environment and its toxicity to human [7, 8, 9].

Some of the commonly used anaesthetics for fish include Benzocaine, Clove oil, Halothane, Isoflurane, Lignocaine, MS-222, Paraldehyde and 2-Phenoxyethanol [10]. Amongst these, Clove oil, Paraldehyde and MS-222 (Tricane methane sulphonate) are the most widely used anaesthetics in aquaculture practices or laboratory experimentation. However, clove oil, due to its cost-effectiveness, efficacy and least side effects is the most preferred anaesthetic in fish. The fish anaesthetics have been subjected to extensive research, but there are still many gaps in our understanding of their effects and efficacy in fish that need to be filled. Several studies have been devoted to investigate efficacy of clove oil on a range of fish species such as *Poecilia vivipara* [11]; *Hyphessobrycon* sp. and *Hemigrammus sp*. [12]; *Oncorhynchus mykiss* [13]; *Silurus glanis* [14].

Snakehead *Channa punctatus* is an important food fish in Indian subcontinent and due to the presence of accessory respiratory organs these fish are hardy and capable of surviving in oxygen deficient water bodies. In most of the studies, the efficacy of the anaesthetic has been assessed based on the induction and recovery time. Understandably, the higher concentration would evoke quick induction and sufficiently longer recovery time but the optimum dose should be such that it may not cause toxicity and disruption of histological architecture of the various organ systems and gets metabolized quickly so as to avoid any detrimental effect on the consumer.

It is evident from literature that, by far, only the relative efficacy of clove oil has been studied in *Channa punctatus* by Matin *et al* 2009 and Chelladurai *et al*. 2013 [15, 16] whereas no attempt has been made to make of the simultaneous assessment of induction and recovery time coupled with the study of the histological architecture of important target organs which are in

direct contact with the ambient environment. This leaves a gap in our knowledge since the reported dose may be toxic causing stress-induced changes in the target organs making the aforesaid dose unsuitable. Hence, the present study has been designed to cover the above aspect and is aimed to study the efficacy clove oil in *Channa punctatus* based on induction and recjhovery time and correlating it with the histological examination of buccal and branchial tissues to assess the descriptive changes, if any to work out the optimum stress-free dose of the clove oil for anaesthetization.

2. Materials and methods

Live specimens of *Channa punctatus* (19±4 g body weight; 12±2.4 cm length) were purchased from the local fish market of Aligarh, and acclimatized in glass aquaria containing de-chlorinated tap water under standard laboratory conditions of temperature and photoperiod (25±2 °C; 12L: 12D) throughout the study period of two months of acclimatization and one day of experimentation. Fish were fed fish feed pellets (Toya fish feed) *ad libitum* on alternate days and the water was siphoned off and replenished with same temperature freshwater. Feeding was stopped 24h prior to the experiment. Fish were treated with $KMnO_4$ followed by 2-3 drops of amphicillin dissolved in 25 litres of water to prevent any bacterial and fungal infection. After 2 months of acclimation, fish were divided into two experimental groups as follows:

2.1 Experiment: Fish were exposed to three different concentrations of clove oil (International Flavours and Fragrances India limited, Chennai) i.e. 50, 100 & 200 µl L^{-1} (five fish were used for each concentration).The experiment was conducted in 60×30×30 cm glass aquarium containing 3L of aerated tap water. Different stages of induction and recovery phases have been formulated based on the criteria given in Weber *et al*. (2009) [2] and Schoettger and Julin (1967) [17] with certain modifications (Table 1). The stage of total loss of equilibrium was considered to be reached at a point characterised by the slow operculum movement to the limit of 20±4 opercular beats per min. The time taken by the fish to pass through all stages of anaesthetization was noted separately for each concentration. After induction, fish were transferred to aquaria containing anaesthesia-free water to measure recovery time.

2.2 Experiment: In second group, fish were also exposed to same concentrations of clove oil as mentioned above. After proper anaesthetization, fish were sacrificed to excise their gill and buccal tissue followed by their sequential fixation in Bouin's and Cornoys fixative, respectively. Five micron thick sections were obtained by a rotary microtome (Leica RM 2125 RTS) and stained with Alcian blue (pH 2.5) and periodic acid-Schiff (PAS) for mucous cells study. The stained slides were observed under Zeiss microscope (Carl Zeiss model Axioscope 40 FL) and images were captured with Zeiss Axiocam ICc3 camera.

2.3 Statistical analysis

The timing of response variables (induction and recovery time) among different concentrations of anaesthetic has been represented as mean ± S.D. Non parametric test was conducted to determine significance of difference of induction and recovery time due to non-normal distribution of the data. Comparisons were made among different concentrations of anaesthetic by using Kruskal-Wallis test and null hypothesis was rejected when $P < 0.05$.

Table 1: Stages of anaesthesia used in the study based on criteria given by Weber *et al*. (2009) [2] and Schoettger and Julin (1967) [17]

Phases of Anaesthesia	Different Stages	Description
Induction phase	Light sedation	Fish swimming, partial loss of reaction to external stimuli.
	Partial loss of equilibrium	Swimming ability stops, slight reaction to external stimuli.
	Total loss of equilibrium	No movement, slow opercular movement.
Recovery phase	Motion perception	Slow and intermittent movement, reaction to external stimuli
	Gain of equilibrium	Recovery of equilibrium, regular opercular movement, quick reaction to external stimuli

3. Results

3.1 Effect of various concentrations of clove oil on the induction and recovery time

The perusal of the result indicates that there is a concentration dependent response in induction and recovery time of clove oil used in the present study i.e. the highest concentration of anaesthetic evoked quickest induction and the longest recovery time (Fig 1). While considering relative efficacy clove oil the concentration of 200µl L^{-1}caused fastest induction (4.47 min) and the highest recovery time (16.88 min) as compared to lowest (50µl L^{-1}) and intermediate (100µl L^{-1}) concentrations (Fig 1). A similar graded response of the various concentrations of anaesthetics is observed in the various stages of induction phase (light sedation, partial loss of equilbrium and total loss of equilibrium) and the recovery phase (motion perception and gain of equilibrium) (Fig 2) where the response is expectedly parallel i.e. highest concentration is quickest to cause unconsciousness and induces longest recovery time.

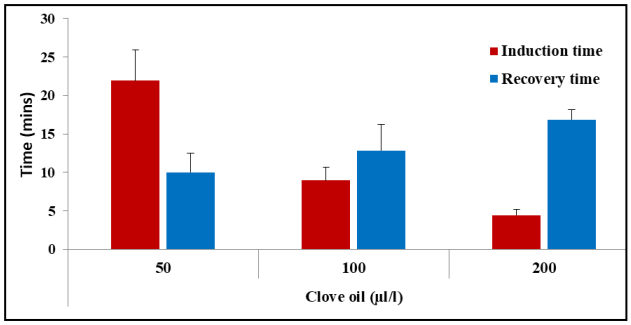

Fig 1: Time required for *C. punctatus* to achieve induction and recovery time at various concentrations of clove oil.

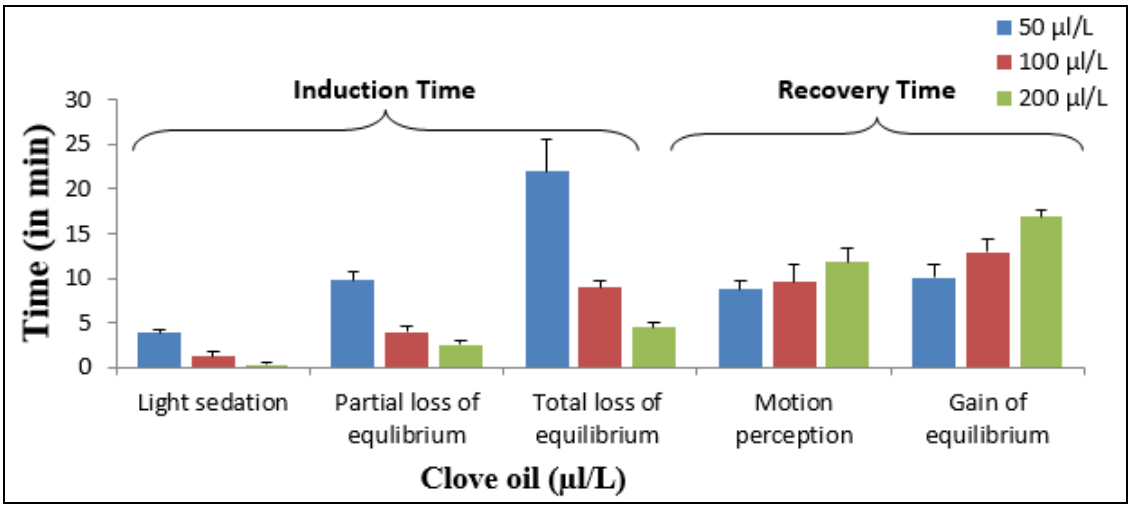

Fig 2: Time required for *C. punctatus* to achieve different phases of induction and recovery at various concentration of Clove oil. Data represents mean ± SD (n=5)

3.2 Effect of various concentrations of different anaesthetics on histology of gill and buccal tissue

Two rows of gill filaments (Primary lamellae) remain attached to gill arch and each gill filament bears a series of alternatively arranged gill lamellae (secondary lamellae) on its either side (Fig 3A). Epithelial layer of gill consists of pavement cells, mucous cells and chloride cells. Mucous cells (MCs) are mostly present on primary lamellae (PL) but occasionally present on secondary lamellae (Fig 3B). Buccal tissue shows epidermis, dermis and muscle layer (Fig 3C). Epidermis consists of epithelial cells and mucous cells which are evenly distributed in buccal epithelium (Fig 3D).

Mucus exudation from mucous cells and lamellar fusion in gills, were the most common microscopic alterations found in the fish exposed to 50 µl L^{-1} and 200 µl L^{-1} concentrations of clove oil (Fig 4). Similarly, buccal epithelium showed dermal lifting and mucus exudation from mucous cells when exposed to 50 µl L^{-1} and 200 µl L^{-1} concentrations of clove oil whereas 100 µl L^{-1} showed only dermal lifting but less prominent than above concentrations (Fig 5).

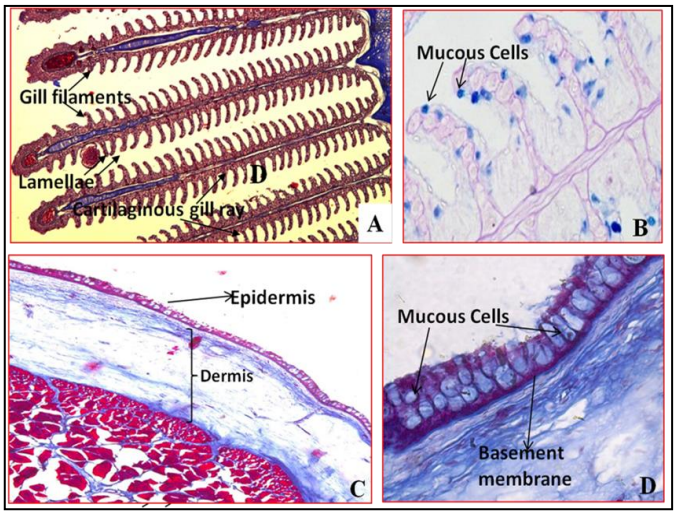

Fig 3: Gill section stained with Hematoxylin and Eosin (3A) and PAS-AB (3B), Buccal tissue section stained with Masson trichome (3C) and (3D) of *C. punctatus*

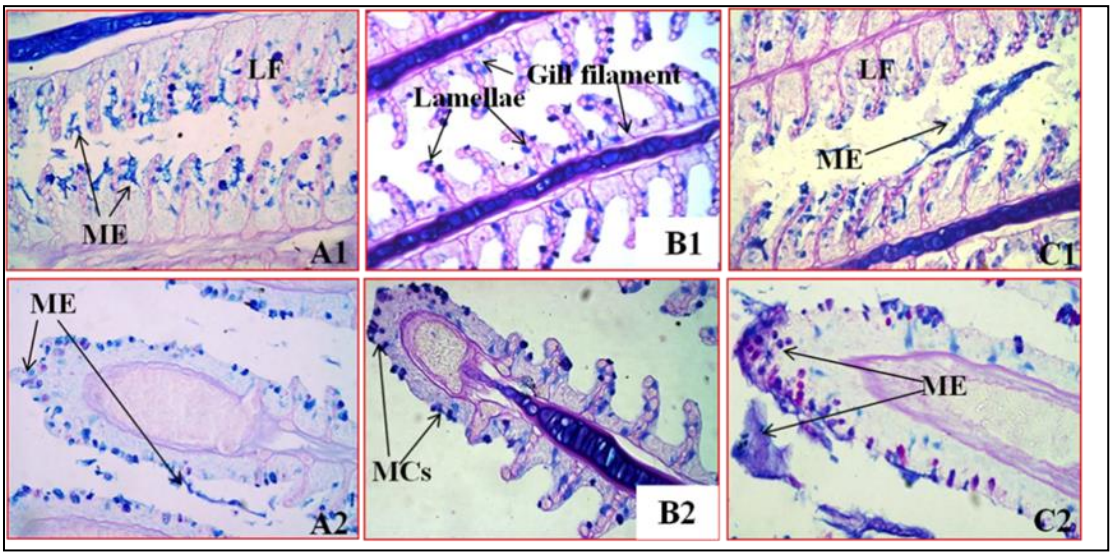

Fig 4: Gill section of *C. punctatus* exposed to different concentrations of Clove oil: 50 µl/l (A1, A2), 100 µl/l (B1, B2) and 200 µl/l (C1, C2). Mucous cells (MCs); Mucus exudation (ME); lamellar fusion (LF). Stain PAS/AB (pH 2.5)

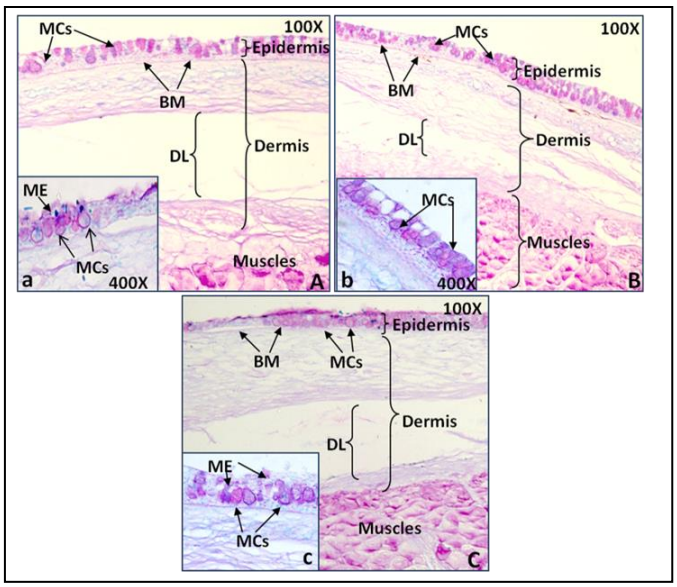

Fig 5: Buccal tissue section of *C. punctatus* exposed to different concentrations of Clove oil: 50µl/l (A), 100µl/l (B) and 200 µl/l (C). Mucous cells (MCs); Mucus exudation (ME); Dermal lifting (DL). Stain PAS/AB (pH 2.5)

4. Discussion

The prerequisite to use using anaesthetics in the efficient management of aquaculture and during laboratory experimentation is to render the fish immobile so as to make it amenable to manipulation without altering its physiology. However, the choice of anaesthetic would not merely depend on the quickest induction and longest recovery time but it is also needs to be ensured that the chosen dose is not so unduly stressful that it may disrupt the normal physiology of the fish and cause histological aberrations in the vital organs. The above fact is clearly borne out from the present study where a maximum dose of clove oil i.e. 200µl L^{-1} seemingly appears most suitable based on rapid induction of anaesthetization and longest period of recovery. However, the study of the histological architecture of gills and buccal epithelium clearly reveals that the dose of 200µl L^{-1} concentration of clove oil is decidedly toxic as is evident from the severe architectural disruption of the above two target organs. Hence, the intermediate dose of 100µl L^{-1} of clove oil is considered most suitable since it does not cause any histological anomalies and the induction and recovery time is also not very drastically different from its highest dose i.e. 200µl L^{-1} which cause mucus exudation, partial lamellar fusion in the gills and dermal lifting of buccal epithelium, all indicators of acute stressful condition of the fish.

It is interesting to note that there is a considerable species-specific variations in the optimum dose of clove oil which causes anaesthetization. Further, literature abounds with quantification of dose of clove oil either on volume or weight basis. In the present study, volume-based optimum dose of clove oil is 100µl L^{-1} (0.01%) in *Channa punctatus* which compares well with that of *Pterophyllum scalare* (90µl L^{-1}) [18], *Hyphessobrycon* and *Hemigrammus* (100µl L^{-1}) [12]. However, towards the higher dose as compared to present study are those reported for *Solea senegalensis* (1000 µl L^{-1}) [19] and several other species where the clove oil dose ranges from 0.03-0.05% [20]. Some other studies have represented clove oil dose in weight/volume basis where the values range from as low as 30 mgL^{-1} in *Channa punctatus* [16], *Cyprinus carpio* [21], *Silurus glanis* [14], to as high as 65 mgL^{-1} in *Colossoma macropomum* [22] and even higher upto 450-900 mgL^{-1} in *Acipenser gueldenstaedtii* [9].

The above information clearly underlines the need to determine the optimum dose for anaesthetization for each anaesthetic on species- basis. Further, the optimum dose should be compared taking into account not merely the induction and recovery time but also the biochemical parameters and histological alterations in the target organs which are in direct contact with the anaesthetic agent during the process of anaesthetization.

5. Conclusion

In summary, the present study clearly established that 100µl L^{-1} is the optimum anaesthetic dose of clove oil in *Channa punctatus* which is relatively safe since it does not cause any architectural disruption or stress-induced changes in the target organs such as gills and buccal epithelium. This information will be of immense applied value to basic scientists, fishery biologists and aquaculturists.

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