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Research Article

Effect of *Origanum vulgare, Eugenia aromatica* and *Cinnamomum zeylanicum* Essential Oils as Welfare Promoters in Gilthead Seabream during Slaughter

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ABSTRACT

The potential of three essential oils, *Origanum vulgare, Eugenia aromatica* and *Cinnamomum zeylanicum* as slaughter anaesthetic agents, is presently assessed. Commercial stunning methods inducing rapid unconsciousness in fish before slaughtering, is urgently required. For now, there is no acceptable method that can ensure kill fish humanely and all the commercially used methods of slaughter for farmed fish include a prolonged period of consciousness. Four slaughtering methods were applied, over-dose of four anaesthetics (three essential oils *Origanum vulgare*, *Eugenia aromatica*, *Cinnamomum zeylanicum* and 2-phenoxyethanol), asphyxia, bleeding and immersion in ice-water, in gilthead seabream (*Sparus aurata* L.). Asphyxia, considered inhumane, but solely used in all wild fish, is also being assessed in Atlantic horse mackerel (*Trachurus trachurus* L.). Plasma cortisol and DNA fragmentation were measured as indicators, to assess stress response and genotoxicity of essential oils. Overdose of all examined essential oils, proved to induce less stress in comparison to asphyxia, bleeding and immersion in ice-water. *O. vulgare, E. aromatica* and *C. zeylanicum* demonstrated higher efficiency in blocking plasma cortisol response to stress in gilthead seabream and lower genotoxic effect, compared to other slaughtering techniques. Asphyxia was confirmed as very stressful slaughtering method in both experimental and wild fish.

Keywords: *Origanum vulgare*; *Eugenia aromatic*; *Cinnamomum zeylanicum*; Slaughter; Welfare; Aquaculture; Fish

INTRODUCTION

The purchase behaviour of the consumers worldwide, reveals that their buying decisions are based on their personal perceptions of value, resulting from a balance between price, quality and social, cultural and educational status [1,2]. Knowledge on livestock production practices and their impact on animal welfare and food quality, are having the most visible influence on consumer's requirements [3]. Consumers expect high quality fish meat, produced with minimal environmental impact, ensuring animal welfare [4].

The EU Council Regulation [5] lays down rules for the killing of animals bred or kept in captivity, for the production of food and other products. Fish are included in the animals that should be spared any avoidable pain, distress or suffering, during their killing and related operations. For the protection of animals used for scientific purposes, according to the EU Council Directive [6], in the process of killing fish, all methods used involve prior sedation, with anaesthetic overdose being the most proposed method. In other cases, slaughtering may occur only on unconscious animals, providing the animal does not regain consciousness before death.

Recommendations on farmed fish are not available, as they present substantial physiological differences from terrestrial animals. Fish are slaughtered and killed in a very different context; therefore, separate standards should be established for the protection of fish at killing. Further initiatives by the Community are based on a scientific risk assessment for the slaughter and killing of fish, performed by EFSA, taking into account social, economic and administrative implications [7]. Furthermore, wide number of fish species is farmed, with an equally wide variety of ecological adaptations and evolutionary developments which react differently to similar situations.

Farmed fish are frequently exposed to a variety of stressors during aquaculture practices which can affect fish performance, growth and survival [8]. Fish quality can be influenced by handling before

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slaughter and by slaughtering methods. Fish welfare is related to physiological stressful procedures which occur during the pre-slaughter and slaughter phase, compromising organoleptic features and commercial quality [9-11]. In the case of farmed seabass and seabream, three slaughtering methods are used under commercial conditions: (a) asphyxia in air; (b) live chilling on ice; and (c) live chilling in ice slurry [12]. Animals should only be killed after stunning and the loss of consciousness and sensibility should be maintained until death [6]. Rapid loss in sensibility and consciousness is a necessary convention to guarantee the fish welfare during slaughtering [13]. Research on the stunning of fish is far less developed than for other farmed terrestrial species. According to the European Food Safety Authority [7], all the commercially used methods of slaughter for farmed fish, include a prolonged period of consciousness during which indications of poor welfare are apparent. Many existing commercial killing methods expose fish to substantial suffering over a prolonged period of time, as there is not a commercially acceptable method that can kill fish humanely [12]. Short term stress induced by normal industrial slaughtering practice, may have a pronounced effect on quality attributes and drip loss, during the shelf-life of pre-rigor filleted farmed fish [14]. Also, fish fillet quality is linked to pre-mortem practices which determine post-mortem muscle biochemical processes [15-18].

During stressful conditions, many physiological changes are involved in a stress response. Cortisol is an important outcome variable that is influenced by stressors [19]. The state of acute stress is better reflected compared to chronic stress; therefore it is a well-accepted acute stress indicator in the plasma [20], as the increase in plasma cortisol is one of the most accepted primary responses to stress [21-23]. Cortisol in fish is measured by different methods, used for quantification of cortisol in biological samples [24]. The role of cortisol in fish physiology and behaviour has been described to assess levels of stress in fish, in terms of growth, sex determination, environmental and ecological impact [25-30]. In farmed fish, stress induced through cultivation practices, has been investigated in some cases and is related to feeding, water supply, stocking density and slaughtering [31-33].

Use of methods that make fish less sensitive to stressful procedures prior to their actual death, is the best way to minimize suffering during slaughter. Treatment with anaesthetic agents is necessary, in order to ensure fish welfare as they are widely used during various farming procedures [34,35]. Anaesthetics could reduce fish stress and improve meat quality, when used in conjunction with other methods of stunning/slaughtering.

Chemical anaesthetics (tricaine methanesulfonate, benzocaine, isoeugenol, metomidate, 2-phenoxyethanol, quinaldine) are included in the most commonly used anesthetic agents for fish [36,37]. However, use of chemicals should be rejected, considering the potential risk for human consumption and more suitable, Food-grade anaesthetics for use in fish should be tested [38].

As aquaculture fish are intended for human consumption, interest in natural products has increased [39]. Essential oils are an alternative option for inducing fish anaesthesia [40] and their anaesthetic efficacy has been assessed. [41-47]. Clove oil (essential oil of *E. aromatica*), which is effective and safe for humans [48], is the most widely used natural product for fish anesthesia [49]. Also, *O. vulgare* and *C. zeylanicum* are the most usually used medicinal herbs [50,51] and their anaesthetic properties have been assessed in case of fish [26].

Essential oils have been used as overdose killing or stunning methods to reduce fish stress and improve fillet quality, separately or in conjunction with other slaughter methods [17,52]. However, the fact that an essential oil is derived from a plant, does not necessarily means that it is fully harmless, as it may have toxic and genotoxic effects [53]. Single-cell gel electrophoresis (SCGE) or comet assay is a useful approach for assessing DNA damage and detection of relevant *in vivo* genotoxicants [54]. This assay offers greatly increased sensitivity for identifying genotoxic agents which induce oxidative stress, both on the cellular and molecular levels [55,56].

There are different welfare implications associated with pre-slaughter procedures and the application of best practices during the several phases of pre-slaughter processes could help to reduce the impact on fish welfare. According to EFSA [7], the development of commercial stunning methods to induce immediate (or rapid) unconsciousness in fish is urgently required.

In the present work, a research to investigate and compare stress response induced by over-dose of three essential oils (*O. vulgare, E. aromatica* and *C. zeylanicum*), over-dose of a chemical anaesthetic (2-phenoxyethanol) and three commonly applied slaughtering methods (asphyxia, bleeding, immersion in ice-water), is presented. Stress response was assessed by measuring physiological indicators (plasma cortisol) and detecting fragmented DNA in hepatocytes, using the molecular technique, single-cell gel electrophoresis (comet assay), which can detect primary DNA damage in individual cells and potential genotoxicity.

MATERIALS AND METHODS

Experimental fish

Gilthead seabreams mean weight 14.97± 5g, originated from a nearby licensed large scale hatchery which supplies seabream and seabass juveniles to various cage farms around Greece (Breeding Code: GR06FISH0008) and transferred to the experimental aquarium facilities (EL-43BIO/exp-01) at the Aquaculture Laboratory of the Department of Ichthyology and Aquatic Environment, University of Thessaly, Greece. Fish were acclimatized in aquarium tanks, supplied with running and aerated seawater (by a recirculation system), for two weeks. Fish were randomly sequenced and allocated in the aquariums. Throughout the experimental procedure, facilities, environmental conditions and feeding were similar to the real farming conditions, meeting criteria of the optimum conditions for the gilthead seabream [57].

Each aquarium was equipped with an independent mechanical and biological filter, a protein skimmer, water pump, ozonizer, thermoregulator, aerator and air diffusers.

Water quality parameters were stable, similar to all aquariums and were monitored routinely with water temperature being maintained at 21.0 ± 1.0°C, pH at 8.0 ± 0.4, salinity at 33 ± 0.5, dissolved oxygen at >6.5 L^{-1}, total ammonia nitrogen at <0.1 mg L^{-1} and photoperiod 12:12 h (light: darkness). Also, artificial pathogen free sea water was used. For the nutrition of the experimental fish, commercially produced dry pellets, appropriate for the fish

size and species (Biomar Hellenic SA), usually offered under real aquaculture breeding conditions and were used once daily by hand. Fish was fasted 24 h before the experiment. Also, wild fish Atlantic horse mackerel mean weight 53.86 ± 7.2 g, caught by fisherman in Pagasitikos gulf Greece, were used to compare experimental slaughtering methods to standard slaughter method for wild fish. Asphyxia is the oldest slaughter method for fish, commonly used for wild fish and is considered inhumane as fish are netted out of water until death.

Slaughtering methods

Four slaughtering methods were applied, over-dose of four anesthetics (2-phenoxyethanol and three essential oils *O. vulgare*, *E. aromatica* and *C. zeylanicum*), asphyxia, bleeding and immersion in ice-water. Three commercial products of pure essential oils used as anaesthetic agents and one commonly used synthetic anaesthetic, were applied for euthanasia. Dosage concentration for euthanasia (Table 1) was administrated in two doses, half dose until deep anaesthesia was induced and the other half until euthanasia was achieved. Seven groups in triplicates for slaughtering methods were accessed in total and five fish were euthanized per group (Table 1). For the outcome assessment, all fish was randomly sampled. The person responsible for outcome and statistical analysis was familiar only with the code of the group and not the treatment. A supervision protocol was applied from the beginning until the end of the study, by the person responsible for the protocol and the Welfare Committee person of the Facility. The person responsible for the animal's care was well trained and qualified in order to recognize adverse effects. Euthanasia of experimental fish was carried out by FELASA accredited scientist.

Blood sample collection and cortisol measurement

Five fish from each of euthanasia group in triplicates were randomly selected firstly anaesthetized and then euthanized as described in Table 1. Blood samples were obtained from the caudal vein using heparinized syringes fitted with 23G needles. Heparin-treated blood was centrifuged at 3000 g for 6 min. Plasma was sampled and frozen at -20°C for the cortisol measurement. Plasma cortisol was measured using commercial Cortisol Elisa Kit by Cayman Chemical, USA (No. 500360) by the FLUOstar Omega microplate readers from BMG LABTECH.

Genotoxic effect of slaughtering methods

To assess the DNA damage *in vivo* due to euthanasia, five fish per group in triplicates were sampled. Liver was removed immediately, and hepatocytes were isolated, as described below. For cell harvesting, hepatocytes isolation was performed by collagenase perfusion, with minor modifications [58,59]. After liver was removed, it was placed in a Hanks balanced salt solution on a glass petri dish. The liver was minced with razor blades and incubated in a 500 μg/mL collagenase solution for 30 min and the cell suspension was filtered through sterile gauze. Cells were dispersed by shaking in a water bath until digested for 15 min. Cells were centrifuged at 2000 rpm for 5 min and were washed two more times with the buffered solution. The isolated hepatocytes were counted using microscope, suspended in PBS before comet assay using Neubauer chamber. Cells viability was determined using trypan blue exclusion test and was found to be greater than 90%.

Comet assay

DNA damage due to euthanasia was assessed by comet assay. The alkaline comet assay was performed according to Singh et al. [60] with minor modifications. Briefly, 100 μl of 0.5% low melting point agarose (LMA) (Sigma, USA) containing 20 μl of cell suspension was dropped to a fully frosted slide, precoated with a 300 μl layer of 0.5% normal melting point agarose (NMA). The agarose was covered with a 22 × 22 mm coverslip and kept at -20°C for 2-3 min to gel the agarose. The coverslips were then gently slid off and a third layer of 100 μl of 0.5% LMA was then applied and solidified on ice. After removal of the coverslips, the slides were then immersed in a freshly prepared lysis solution (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, with 1% Triton X-100 and 10% DMSO added just before use) at least for one hour at 4°C. The slides were removed from the lysing solution and placed on a horizontal gel electrophoresis unit filled with a freshly prepared alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) to a level 0.25 cm above the slides. The slides were allowed to set in this buffer for 25 min to allow unwinding of DNA before electrophoresis which lasted for 20 min. All procedure was conducted in the dark to prevent additional DNA damage. The slides were then rinsed with neutralization buffer (0.4M Tris buffer, pH 7.5) three times.

Scoring of DNA damage using image analysis

The DNA in the nuclei was stained by 20 μl ethidium bromide (20 μg/ml) in distilled water solution on each slide and then the slide was covered with a coverslip. DNA was analyzed in a dark room, using a fluorescence microscope at magnification 40x with an excitation filter of 525-570 nm. Images of 150 randomly selected nuclei (fifty counts on each triplicate slide) were analyzed for each sample. DNA migration was analyzed by image analysis and CASP software, determining the tail moment (TM).

Statistical analysis

For sample size calculation of the experimentation GRANMO

Table 1: Slaughtering methods applied in fish.

Anaesthetic agent		Concentration for euthanasia (μL×L^{-1})	Trademark
Essential oils derived from medicinal plants	*Origanum vulgare*	100	STYX
	Eugenia aromatica	100	STYX
	Cinnamomum zeylanicum	100	STYX
Synthetic anaesthetics	2-phenoxyethanol	1000	SIGMA-ALDRICH
Asphyxia	Fish were netted out of water until death		
Bleeding	Fish were exsanguinated by cutting all gill arches		
Immersion in ice-water	Hypothermia through chilling on ice slurry		

(V.7.12) software was used. Data obtained from each method were analyzed separately and the effect of all slaughtering methods was compared. Cortisol levels and genotoxicity TM data were expressed as the mean ± standard error and analyzed using one-way ANOVA followed by Tukey post hoc test. All data were analyzed using SPSS-17 statistical package while differences were considered significant at $p<0.05$.

RESULTS

The concentration of plasma cortisol was different between the experimental groups (Figure 1). Significantly high level was recorded in case of bleeding, icewater and asphyxia, without statistical differences between these three experimental groups. Use of anaesthetic agents reduced the plasma cortisol levels. In case of essential oils, the values were higher compared to chemical anaesthetic phenoxyethanol, in which the significantly lower ($p<0.05$) cortisol level was recorded. Asphyxia, as slaughtering method, recorded similar results between the experimental and wild fish groups.

Genotoxicity was accessed by DNA migration (tail moment) which was recorded during application of different slaughtering methods (Figure 2). According to the results (Figure 3) overdose of anaesthetics induced significantly lower DNA migration ($p<0.05$) compared to bleeding, icewater and asphyxia. No differences were observed between the DNA migration induced by overdose of essential oils *O. vulgare*, *C. zeylanicum* and *E. aromatica* and chemical anaesthetic agent (2-phenoxyethanol). Also, no differences were recorded, comparing the DNA strand breakage of the experimental and wild fish groups, in case of slaughtering by asphyxia.

DISCUSSION

In accordance to E.U. regulations, killing of farmed animals for food production or for scientific purposes must be void of pain, distress and suffering. During aquaculture practices, fish performance, growth and survival are affected by stressors [8]. Stressful conditions induced by slaughtering methods can affect the commercial quality of the end product. The development of

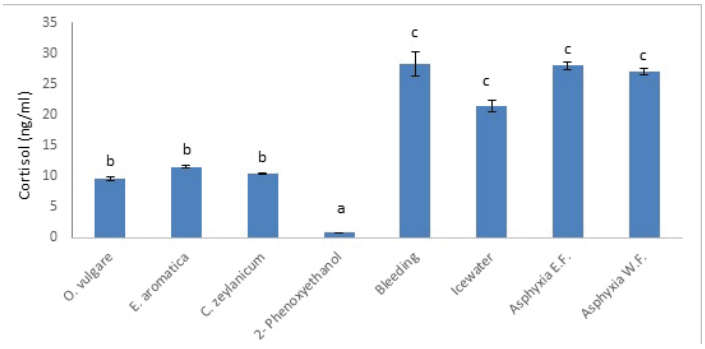

Figure 1: Effect of different slaughtering methods on plasma cortisol level (X ± S.E.). Letters a, b and c indicate significant differences between treatments (ANOVA; *P*<0.05). E.F.: Experimental Fish, W.F.: Wild Fish.

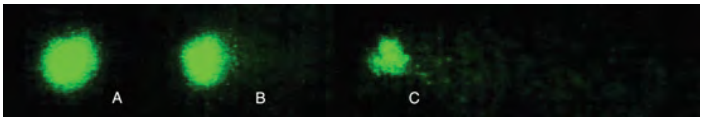

Figure 2: Different levels of DNA damage. (A) Cell's nuclei with normal intact DNA (B and C) Nucleus with medium and severe damaged DNA.

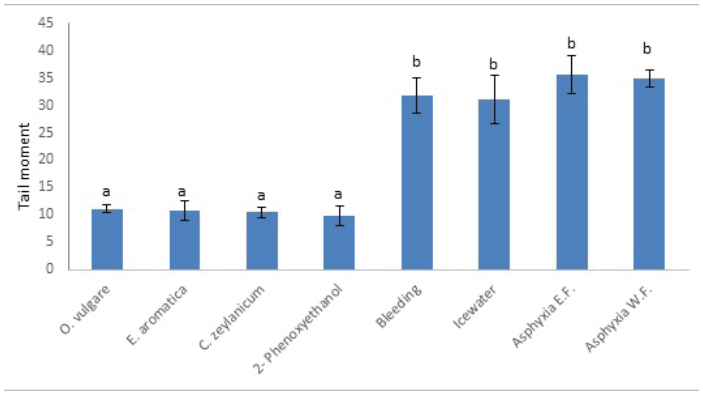

Figure 3: DNA migration represented as mean tail moment ± S.E. from hepatocytes after applying different slaughtering methods. Letters a and b indicate significant differences between treatments (ANOVA; *P*<0.05). E.F.: Experimental Fish, W.F.: Wild Fish.

stunning methods leading to immediate or rapid unconsciousness of fish before slaughtering, is an emerging necessity [10,12,61]. As different fish species react differently to similar situations, stress induced during slaughtering ought to be species specific and separately investigated [7]. In reference to EFSA [12], animals should only be killed after stunning. Loss of consciousness and sensibility should be rapid, in order to guarantee the fish welfare during slaughtering and maintained until the death of the animal [13]. A prolonged period of consciousness, prior to killing, is characteristic of all commercial slaughtering methods presently used in aquaculture, since no other alternative acceptable humane methods for this are practiced [12].

Induced stress and genotoxicity of slaughtering, by over-dosing fish with three essential oils, *O. vulgare*, *E. aromatica* and *C. zeylanicum*, was presently assessed. Their effects were compared with over-dosing with a widely used chemical anaesthetic (2-phenoxyethanol) and three commonly applied slaughtering methods (asphyxia, bleeding, and immersion in ice-water). Anaesthetic overdose is the proposed method for protection of fish used for scientific purposes, in the process of killing, according to the EU Council Directive [6]. The use of essential oils is an alternative option for inducing fish anaesthesia [62], as well as other suitable food-grade anaesthetics [38].

Interest in natural products with anaesthetic properties has increased, as aquaculture fish are intended for human consumption. Extracts derived from medicinal plants have low cost, easy accessibility, efficacy and are environmentally safe [39,63]. According to stress indicators presently measured, plasma cortisol and DNA fragmentation overdose, of all essential oils examined, proved to induce less stress, as compared to asphyxia, bleeding and immersion in ice-water. Essential oils extracted from *O. vulgare*, *E. aromatica* and *C. zeylanicum*, have an anaesthetic effect similar to that of chemicals [44], possessing the characteristics of the ideal anaesthetic [64]. Clove oil is the most widely used natural product for fish anesthesia [11,52,65-67]. On the other hand, asphyxia is considered as one of the most stressful slaughtering methods, increasing haematological parameters, glucose levels, lactate and cortisol response [68,69]. Furthermore, bleeding methods don't lead to rapid death or unconsciousness, exhibiting extreme adverse reactions, such as vigorous escape attempts and wide opening of the mouth and gills [70]. Cutting the gills is not effective in provoking death rapidly [71], even though it is considered more efficient than

cutting the caudal vein [70]. Finally, chilling fish is the oldest and simplest method used in inducing anaesthesia [72,73]. Chilling results in some sort of sedation when fish are placed in ice slurry, as their examined responsiveness is low. Rapid temperature changes have been assessed to cause stress, [74], therefore fish may be subjected to stress between the times they are placed in ice slurry to the induction of sedation [75].

The medicinal plants *O. vulgare* and *C. zeylanicum* have traditionally been used by folk medicine for their medicinal properties, while their anaesthetic effect has been recently assessed [76,77]. Herbal essential oils or extracts have been found to induce anaesthesia in fish, with positive health effects [39]. Cortisol fluctuation is an important physiological change which is involved in a stress response [19] and one of the most accepted primary responses [22] reflecting acute stress [20].

The ability of an anaesthetic agent to mitigate cortisol stress response is a desirable characteristic [78]. *O. vulgare*, *E. aromatica* and *C. zeylanicum* used in the present study, demonstrated high efficiency in blocking the plasma cortisol response to stress in gilthead seabream, as in all examined cases, plasma cortisol level was significantly lower compared to other slaughtering techniques. The clove oil preventive behaviour upon cortisol release in *Salmo salar*, has been discussed before by Iversen et al. [79] indicating that there is a blockage of sensory information transmission to the hypothalamus, not triggering the hormonal cascade. Clove oil is one of the most commonly used anaesthetics in the aquaculture industry, as it is an effective anaesthetic without negative side effects on the fish [51]. Clove oil is one of the optimal anaesthetics in relation to cortisol response [80-82]. Prevention of cortisol release has been also achieved by *Aloysia triphylla* and *Lipia alba* [83,84], Origanum oil and eucalyptus oil [85].

In the present study the chemical anaesthetic 2-phenoxyethanol presented significantly higher cortisol-blocking properties, recording the lowest plasma cortisol levels. A similar approach was described by [86] who found that the essential oil of *L. alba*, induced a higher cortisol level compared to 2-phenoxyethanol in gilthead sea bream. According to Golomazou et al. [46], 2-phenoxyethanol induces deep anaesthesia in a significantly shorter time in comparison to *O. vulgare*, *E. aromatica* and *C. zeylanicum*. This could explain the low plasma cortisol levels, as the effect of the aneasthetic on plasma cortisol is dose dependent, determining the time of anaesthesia induction [83-88]. During treatment, the anaesthetic agent is dispersed in the water and absorbed across the gills, the time of anaesthesia induction depending on, the amount of anaesthetic absorbed by the fish and their exposure time to the anaesthetic bath [35].

The fact that an essential oil is derived from plants does not necessarily mean that it is fully harmless, as it may have dose dependent toxic effects [53]. Essential oils may be safe in low concentrations but display toxicity at high concentrations [90]. DNA strand breakage acts as a biomarker of genotoxicity in fish and other aquatic species [91,92], sustaining competitive advantages compared to other genotoxic assays [93]. Herein, genotoxicity was assessed by *in vivo* comet assay, which is considered mature enough to ensure reliable detection of relevant genotoxicants, included in the ICH S2(R1) guidance [54].

Fish exposure to *O. vulgare*, *E. aromatica* and *C. zeylanicum* induced DNA migration in hepatocytes, indicating genotoxicity. DNA strand breakage has been recorded before at fish exposure to essential oils, by comet assay [94]. Clove and origanum oil, are known to act as irritants [95]. Clove oil is related to oxidative DNA damage [96] and is known as a cytotoxic compound for prokaryote and eukaryote cells [97-100]. Also, cytotoxic effect of cinnamaldehyde on tumor cells has been proved [101-104], while its antitumor properties are related to its antioxidant activity [105]. The variability of the essential oil composition is stated to be an equally important factor which affects their cytotoxic properties, while alcohol, aldehydes and phenolic constituents are the main cytotoxic agents [106].

However, in the present study, the genotoxic effect of the essential oils tested, proved to be significantly lower as compared to asphyxia, bleeding and immersion in ice-water, as tail moment values in hepatocytes of all anaesthetic groups were lower. DNA damage recorded in the present study was higher, compared to previous studies [46] and this is probably related to anaesthetic overdose. Clove oil genotoxic effect is dose-dependent [67]. This activity has been mainly attributed to eugenol, inducing mutations in eukaryotic cells, but the mechanism involved is not well understood [107]. However, *O. vulgare*, *E. aromatica* and *C. zeylanicum* may act as antioxidants and anti-inflammatory or pro-oxidant agents, depending on their concentration, indicating a potential geno-protective role for essential oils [46,108-112].

CONCLUSION

FAO predicts that by 2030, sixty-two percent of consumed fish will be of aquacultural origin. Therefore, developing commercial stunning methods that induce rapid unconsciousness in fish before slaughtering in aquaculture, presents an urgent requirement. According to stress indicators presently measured, overdose of all examined essential oils proved to induce less stress compared to asphyxia, bleeding and immersion in ice-water. Compared to other slaughter techniques the use of *O. vulgare, E. aromatica* and *C. zeylanicum* demonstrated high efficiency by blocking the plasma cortisol response to stress in gilthead seabream and had a lower genotoxic effect. In the present study, the three essential oils examined can possibly be developed to potential anaesthetic agents promoting welfare in fish during slaughter. However, the implication caused by using essential oils as commercial food-grade anaesthetics and their link in yielding a spicy, pungent taste and possibly modifying the flavour of fish fillets, must be further investigated.

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Conflicts of Interests

The authors declare no conflict of interest.

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