Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.4, No.19, 2014

Stress Reducing Substance of *Ageratum conyzoides* and Its Application to Koi Carp (*Cyprinus carpio*) Transportation

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Abstract
The aim of the research is to determine the potential of essential oils from the *Ageratum conyzoides* leaves as a stress reducing substances that can be used in the transportation of koi fish. The research consists of several stages, that is (1) extraction of *A. conyzoides* leaves to obtain the essential oil chemical composition analysis, qualitative and quantitative analysis as well as essential oil solubility in water, (2) biological activity assay of essential oil from *A. conyzoides* leaves to koi fish, and (3) biological activity assay of essential from *A. conyzoides* leaves as a stress reducing substances at different concentrations of the stress response (tachiventilation, blood glucose levels, levels of plasma cortisol, levels of Na^+ and Cl^- in blood plasma). The *A. conyzoides* leaves has a chemical composition of chromen, terpenes, coumarin and phenol. The 24 h LC_{50} and safe concentration values are 34.047 and 10 ppm, respectively. The next results of the study showed that the concentration of essential oils given (5 and 10 ppm) and time of observation (0, 0.5, 1, 2, 4, 6, 8 hours and 1 hour after transportation) influence on the measured stress response. Results of the analysis also showed that there is an interaction between a given concentration, time of observation and mortality. Interestingly, based on the analysis of the MARS program were obtained the equations Y = 15.538 + 29.415 * BF1 - 12.163 * BF3 - 4.859 * BF4. The *A. conyzoides* leaves essential oils have chemical components that work on the central nervous of fish to affect the response of primary, secondary and tertiary from fish.
Keywords: transportation of koi fish, stress reducing substances, *Ageratum conyzoides*

1. Introduction

The trend of the volume of exports ornamental fish Indonesia shows that in 2007-2011 increased by 11.6%, while increasing the value of fish exports 29.38%. The value of Indonesia's exports ornamental fish seeds into Asian countries amounted to 161,817 kg (Ministry of Marine and Fisheries, 2011). An increase in the business of aquaculture developments cause to demand fish also raise so that efforts are required transportation. Transportation is the activity of transporting fish from one place to another place in a zone or area in the form of lives either the size of a seed, consumption and brood stock.

The fish farmers generally do fish use the closed transportation delivery because it has advantages in terms of delivery time is relatively longer for long distance and amount of fish or seed sent a relative can be adjusted without consuming a lot of room. However, the delivery of fish through transportation often causes death. The death of the fish not only occurred during transportation but also happening on post transportation, so the fish life after the transportation is a critical period as the fish have been transported. Deaths from transport and handling transportation reached 30-40%. The fish are recovered after the transportation takes more than 96 h, if at the time the fish have not recovered then shows the next stressor can cause death (Urbinati & Carniero, 2006). Ross & Ross (2008) reported that the problems encountered in transport are the occurrence of stress on fish caused by mechanical handling, temperature changes and changes in water chemistry. Wedemeyer (1990) added that the change of water quality during transport in general due to a change in temperature, pH, dissolved oxygen, accumulation of residual metabolites materials coupled with the stress that comes from the procedural handling of the transport activity.

The stressor causes the fish gives a response so that the body remains in a condition of homeostasis (Barton, 2002). On aquatic animals, especially fish, its influence is apparent in the external and internal parts of the body. The characteristics of external signs of stress on the fish is swimming movements were random, real tachiventilation change and change in body color became more dark or pale (Ross & Ross, 2008). Internal response to stress may be the primary response, secondary and tertiary. As for the primary response, namely the involvement of neuroendocrine responses includes release of catecholamine from the network chromaffin (Reids *et al*., 1998) and stimulation along the hypothalamus-pituitary-interrenal (HPI Axis) to release the corticosteroid hormone (Barton, 2002).

In a few seconds the sympathetic nerves and the combination action of adrenaline prepared body tissues with β-adrenoreseptor for the fight to flight accelerate the heart and respiration rate, raise blood pressure and blood flow to the muscles and stimulates the release of glucose. The release of corticosteroids can stimulate gluconeogenesis in fish, stimulates lipolysis, speed up the transport of ions which are the important properties of

fish to regain osmotic balance with the environment (Pickering, 1993). These mechanisms are included in the secondary response. Tertiary response can occur and lead to overall performance aspects of animal, including changes in growth, metabolic scope for activity, behavior and survival rate (Iwama *et al*., 2006).

Fish physiology response of existing stressor if not be solved by good will affect the survival rate of fish. Therefore it needs to be an effort to provide materials on media transport so as to reduce the effect of stress on fish (stress-reducing substance) when transported. In the field of fisheries, has been widely known chemicals provided when fish are transported that aims to reduce stress, reduce hyperactivity and movement causes trauma during handling so as to minimize damage to integument disorders associated with osmoregulation, as well as lowering metabolism i.e. decrease in oxygen consumption and decrease waste or waste product (Neiffer & Stamper, 2009).

Sukarto & Wibowo (1993) reported that chemicals that are used to reduce stress at the time of fish transportation, such as MS-222 (Trichain methasulfat) and Quinaldine (2-4 Methycynolon) and nitrofurazone as bacteriostatic. However, these materials are expensive and toxic. The chemicals also have the effect of residue on organisms. It was reported that residue of MS-222 in the body of the fish for more than 30 days. Ross & Ross (2008) showed that the sperm motility of brooktrout dropped on fish which it used MS-222 with a dose of 19 mg/L. Based on these phenomenon, it is looking for that alternative materials needed as a stress reducing substance that comes from natural materials which are easily obtained, and cheap price.

Natural materials, such as clove oil, are commonly used as stress reducing substance. Clove oil, in the field of fisheries is used as an ingredient of anesthesia during handling and transport. At carp, clove oil effective as anesthesia at 25-100 ppm (Wagner *et al*., 2003). Inoue *et al.* (2005) showed the *Brycon cephalus* fish which are transported using clove oil can decrease plasma cortisol, glucose levels and the plasma ion balance (Na^+, Cl^-, and K^+) at a concentration of 5 mg/L. According to Bhuiyan *et al.* (2012) clove oil has a main component of 74.3% of eugenol. The components can it penetrates on blood brain barrier and acts on nervous systems (de Sousa, 2011). However, crude clove oil has problems at the smell and taste. Furthermore, the nature of eugenol is incrusting of epithelia gills and it can block diffusion of gases possibility (Sladky *et al*., 2001), or the presence of necrosis on the gills of exposure to a dose of eugenol in a recurrent manner (Neiffer & Stamper, 2009).

Ageratum conyzoides in Indonesia is known as bandotan plant, that is the wild plants and better known as plant buggers (weeds) in gardens or the fields. Bandotan plant (*A. conyzoides*) having bioactivities reportedly, it is effective against cardiovascular, having activities of analgesic, inflammatory, antibacterial, reproductive, anti fungal, insecticides, antihelmintic and allelopathy (Kamboj & Saluja, 2008). Ekundayo *et al.* (1988) and Vera (1993) are also indicating that the volatile oil of *A. conyzoides* having monoterpen content and sescuiterpen. Based on these results, it was necessary to know the composition of *A. conyzoides* as a stress reducing substances with regard to have on various response stress and its application on various fish related density and different time of fish transportation.

2. Materials and Methods

This research is experimental research that requires some research steps as follows: (1) consisting of extraction of *A. conyzoides* leaves to get essential oil, analysis of chemical composition (qualitative and quantitative) and solubility of volatile oil in the water; (2) assay of biology activity from the volatile oil of *A. conyzoides* leaves related to fish with the purpose of acquiring survival rate or mortality, the value of 24 h SC (safe concentration, 24 hours) and 24 h LC_{50} (median lethal concentration, 24 hours); (3) activity test of the volatile oil from *A. conyzoides* leaves as a stress reducing substances on various concentrations of stress responses, that is tachiventilation, blood glucose and cortisol plasma levels, Na^+ and Cl^- in blood plasma, survival rate of koi fish (*Cyprinus carpio*) related transported for 8 h.

2.1 Preparation of materials

Material used was *A. conyzoides* leaves that taken at in the Tunjung Village, Lowokwaru, Malang. Identification of *A. conyzoides* leaves were worked by UPT Balai Konservasi Tumbuhan Kebun Raya Purwodadi LIPI Pasuruan. Collecting, drying and extracting of *A. conyzoides* leaves was performed by methods of Gunawan & Mulyani (2004). Material that has been ready then continued with a extraction to acquire volatile oil by steam of water distillation, that comparisons between leaves and water is 1:5.Water is heated for producing vapor that carries the fraction of steam with material aromatic. Steam is cooled, and then destilat is accommodated. A volatile oil will float on the hidrosol layer. Furthermore, it can be extracted using n-hexane by comparison 2:1, and whipped in a separating funnel as well as three times repeatedly. The result of separation then evaporated using rotary vacuum evaporator to separate between n-hexane and a volatile oil at a temperature of 70^{o}C, 40 rpm. A volatile oil accommodated then dried by the addition of Na_2SO_4 anhydrous then filtered (Tripathi *et al*., 2008).

2.2 Analysis of the component parts of a volatile oil

The volatile oil of *A. conyzoides* leaves obtained then analyzed its chemical composition qualitative and quantitative, similarly with clove oils. 5 mL of volatile oil sample dissolved in 500 mL of n-hexane and injected

1 µL into GC-MS. Furthermore, each mass spectra and peak confirmed by reference of mass spectra with hard-wired intoWiley Program version 8.0. Specifications and conditions of GC-MS instruments using Agilent 6980N Network = GC System with auto sampler, Agilent 5973 inert MSD as a detectors, column using J&W Scientific, HP_55% phenyl methyl siloksan 30 m, 0.32 mm, 0.25 µm, inlet = split 1:100, 280^{o}C, programmed oven: 50^{o}C (1min) -> 2 ^{o}C/min ->100^{o}C->5^{o}C/min->290 ^{o}C (10 min), flow in a column = 1.4 mL/min (constant), Aux = 280 ^{o}C, MS Quad = 150 ^{o}C, MS Source = 230 ^{o}C, scan mode = 30-600 amu, solvent delay = 3 min. Test the solubility of a volatile oil in water was perfomed by method of Shriner & Shriner (1980) with minor modification.

2.3 Biological activity of the essential oils of A. conyzoides leaves

Koi fish (*C. carpio*) are used for research are selected for health condition and uniformity of size. Biological activity test was performed to obtain the value of mortality, the 24 h SC and the 24 h LC_{50}. Preliminary research results obtained that koi fish can suvive at < 100 ppm, so that the test is done by making a solution of different concentration started 10 until 90 ppm and compared to a negative control (blanko). After the solution is created, it is in each container solution is given a total of 10 koi fish and observed for 24 hours. Fish mortality was obtained by counting the number of fish that die divided the entire number of fish kept multiplied 100% (Effendi, 1997). The mortality rate can be used as data in the counting of the 24 h LC_{50}, and obtained the value of 24 h SC. This research was also conducted on clove essential oil that is used as a positive control. Next, the study is done by making solution various concentrations still in extent safe concentration to be a medium of fish transportation.

2.4 Transportation of koi fish

Fish did not be given feed for 24 hours before being transported. The number of fish that are 10 fish for each 3.3 L of plastic bag. Water media used is water that has containing a volatile oil of *A. conyzoides* leaves, then given the addition of oxygen with comparison water and oxygen by 1:1, next plastic fastened with rubber and put into a styrofoam box. Box styrofoam before use conditioned ancients at temperature 20^{o}C by means giving ice cubes that has been plated by paper to not directly touching a plastic bag containing fish. In this research, fish transportation done for 8 h with a closed system. Water quality parameters measured were pH, dissolved oxygen and ammonia.

2.5 Observations of tachiventilation

Observation of tachiventilation done by observing open and shut down of fish operculum (Ross & Ross, 2008). Duration of tachiventilation observation is 3 min which it done before fish transported and every 0.5, 1, 2, 4, 6, 8 h of fish transportation, and 1 h after transportation.

2.6 Measurement of blood glucose levels and cortisol

Fish blood is needed in the form of fresh and plasma. Fresh blood is needed for examination of blood glucose, while blood plasma is needed for cortisol levels and examination of Na^{+} and Cl^{-}. Blood is drawn from the heart using a microcapillary (already contain heparin). Measurement of blood glucose levels is done by direct blood shed on a glucose test strip. The provision of blood plasma is carried out by holding the blood in the microtube. Blood samples left at room temperature for 1 hour and then stored in the refrigerator overnight. Furthermore, it was centrifuged at 3000 rpm for 30 min (Kubilay & Ulukoy, 2002). Measurement of blood glucose levels used Blood Glucose Monitoring System, while measurement of cortisol levels conducted by the method of Enzyme-Linked Immunosorbent Assay (Sink *et al*., 2008).

2.7 Measurements of Na^{+} and Cl^{-}

Measurements of Na^{+} and Cl^{-} is done by inserting a 0.5 µl of blood plasma into the sample cuvet then put in a chemical analyzer using ion selective electrode method (Percin *et al*., 2010).

2.8 Data analysis

24 h LC_{50} values retrieved from the data analyzed with mortality using probit analysis of SPSS program. Physiological response data for survival rate, tachiventilation, blood glucose, cortisol, Na^{+} and Cl^{-} analyzed by univariate factorial, as a factor is the concentration and time of observation. If there are interactions tested with ANOVA. If there is a real difference then tested with Tukey test. To find out the relationship between the transportation time, metabolite concentration, fish density and mortality , it was performed by a test using MARS (Multiple Adaptive Regression Splines). All analysis is performed with the SPSS program.

3.Results and Discussion

According to Ekundayo *et al.* (1988) hydrodestillation of *A. conyzoides* leaves will generate volatile oil which is reddish orange colored by terpenes compounds are very sensitive to the process of oxidation and polymerization by light and water in oil (Guenther, 1987). Results of the yield of essential oil of *A. conyzoides* leaves from Indonesia is 0.3%. Vera (1993) pointed out that the most essential oil obtained from Cameroon amounted to 0.7%, while the lowest of Nigeria by 0.06%. Okunade (2002) reported that essential oil content are varying between 0.11-0.58%, whereas it depend on climate, season and geographic conditions, periods of harvesting and distillation techniques (Tripathi *et al*., 2008).

Qualitative and quantitative analysis of the essential oil is done by using GC-MS (Gas

Chromatography-Mass Spectrophotometer) aims to determine the chemical composition of the essential oil from the *A. conyzoides* leaves and cloves (Figure 1.). Matching the mass spectrum of GC-MS instruments can be done by looking at the similarity of the spectra is indicated by the value of the qualifier (Q), the value of the qualifier of at least 80% accepted. The mass spectra results suggest that eugenol from clove oil used as controls when compared to the library have a similarity of values shown by Q of 98% and in mass spectroscopy can be known molecular weight values of compounds were analyzed and compared with the mass of eugenol standards. Eugenol has a molecular weight of 164 m/z with the molecular formula $C_{10}H_{12}O_2$ (Figure 2.). Next, the results of mass spectra from ageratochromen (Precocene II) of *A. conyzoides* when compared to the library have a similarity of values shown by Q of 93%. Ageratochromen has the molecular formula $C_{13}H_{16}O_3$ (m/z = 205). (Figure 3.) The result of the fragmentation of eugenol which is the mass of m/z (eugenol): 164 as M^+, 149 (M-CH_3), 131 (M-(CH_3 + H_2O)$^+$, 103.91 ($C_7H_7^+$). This is in accordance with the result of the fragmentation of eugenol which is 164 m/z as the eugenol in the form M^+ and showed a group of ether being detected from the release of CH_3 produced by ether. It was detected hydroxyl groups with a further release of H_2O that it was shown m/z 131, and alkenes with a loss $H_2C = CH_2$ producing m/z 103 (Figure 4.)

The largest component of oil of cloves is eugenol, caryophyllene, α-caryophyllene, caryophyllene oxide, and naphthalene (Table 1.). Based on the results of the analysis of the composition of the essential oils of *A. conyzoides* leaves is the chromen 79.9% consists of 3 components, followed by the terpenes of 15.1% consisting of 12 components while the coumarin of 2.1% and amounted to 1.4% phenol groups, each consisting of 1 component (Table 2.) Chromen (benzopyran) is a heterocyclic ring consisting of a benzene ring fused with pyran (Thomas & Zachariah, 2013). Compounds from the chromene derivative of chromone, especially 6-amino and 4-acetamido have been reported to have anti-depressant properties, analgesic, and antipyretic (Kamboj & Saluja, 2008). Precocene compound known to many as an insecticide activity due the influence of hidrolysis inhibition of acetylcholine thereby inhibiting the nerve impulse transmission of nerve impulses, which hampered the complete and cause death in insects (Aboua *et al*., 2010). Precocene II has the ability to lower glucose levels in blood (Nyunai *et al*., 2010). Adebayo *et al*. (2010) also reported that precocene II was probably the active phytochemicals that are responsible for the activity of hypoglycemic. Hypoglycemic effect caused by the potential of insulin in plasma increases insulin secretion by the pancreas β or by releasing insulin that is bound. The coumarin found in leaf essential oil bandotan is 2 H-1-Benzopyran-2-one. Coumarin (benzopyrone) is a toxic chemical component and many found in various plant (Akah *et al*., 2010). Coumarin was blocked against cancer (Amadi *et al*., 2012). Coumarin can inhibit the synthesis of vitamin K in the liver, which is responsible for the coagulation factors II, VII, IX and anticoagulant proteins C and S (Akah *et al*., 2010). 2-Methoxy-4-vinyl phenols contained in *A. conyzoides* is the phenol. 2-Methoxy-4-vinyl phenols with chemical formula $C_8H_{10}O_2$ according to EFSA (2012) can be used as flavouring in both human and livestock feed but in large amounts can cause irritation of the respiratory tract. Monoterpen is a key component in many essential oils that have properties inhibit the growth of competing plants and can work as an insecticide. Sesquiterpen is a compound that works as an insect repellent and insecticide, growth stimulants and some other as fungicides (Robinson, 1995). Essential oils have a wide range of biological activities such as anxiolytic, anticonvulsant, spasmolytic and antinociceptive (de Sousa, 2011).

Based on the results of the analysis of solubility in water, essential oil of clove and *A. conyzoides* is 111.7 ppm. Neiffer & Stamper (2009) said that material that is given in the form of immersion should be that water soluble or using a solvent water soluble as the carrier.This is in accordance with the result, because the range of the solubility of a volatile oil in the water is still above concentration of solutions test (Table 3).

Biological assay activity of essential oil from *A. conyzoides* to koi fish for 24 hours at this stage aims to get the value of mortality, the 24 h LC_{50} values, and the safe concentration. Koi fish mortality occurred in the treatment of 20 ppm to 90 ppm, while 100% mortality occurred in the treatment of 50 ppm to 90 ppm (Table 4.). Based on probit analysis, values of 24 h LC_{50} is 34.047 ppm, whereas the value of the safe concentration (both clove oil and *A. conyzoides*) is 10 ppm. Mortality during transport can be caused by prolonged stress on the fish. According to Floyd (2010) reaction to signs of danger (alarm reaction/respon fight or fly) on the fish are marked with the following mechanisms: the first mechanism is the increased blood sugar caused by the secretion of hormones from the pituitary gland, kidney sugar is stored as glycogen in the liver will be metabolized. This condition will result in an energy reserve that are prepared by fish for emergency action. The second mechanism is a process of osmoregulation that occurs because of a change in the metabolism of the mineral. This situation led to a fresh water fish will tend to soak up the excess water from the environment (overhydration) and a sea fish tend to lose a lot of water to the environment (dehydration). Condition of disorders such as this requires extra energy used to maintain osmoregulasi. The third mechanism is the increased respiration, increased blood pressure and red blood cell reserve is released into the circulation. The fourth mechanism is a response to the presence of inflammation that is stymied by the presence of hormones from glands in the kidneys. The body retains power (endurance/body Resistence) on fish, occur if are able to adapt to stressful conditions for limited periods of time. During this period the fish may look normal, but can spend the energy reserves because it needs

extra for the State. If that can not be corrected then there will be fatigue (exhaution), on this condition and energy reserves had been spent on adaptation fails because the stress was too strong and persist for a long time. Dissolved oxygen and pH were still in a range that can be tolerated by koi fish. The water temperature during assay activity reaches 31^{o}C (this value exceeds the optimum temperature for the growth of koi fish). The observed of highest value of ammonia is reaching 0.27 ppm. It means that water quality of treatments can be tolerated by koi fish. (Table 5.) Furthermore, koi fish are transported for 8 h, which it did not occur death until concentrations of 10 ppm at the treatment of *A. conyzoides* essential oil. On the contrary, the highest concentration of clove oil in the water medium for 8 h, without causing deaths, occurred at 5 ppm (Table 6.).
The results showed that during 8 h of koi fish transportation, average value of tachiventilation decreased until the final time of the observation, whereas blood glucose levels in all treatments increased compared to the value at the beginning of transportation (Table 10). At 0.5 h, the highest cortisol values contained on the B treatment (essential oils of *A. conyzoides* with concentrations of 10 ppm) different significantly with A treatment (essential oils of *A. conyzoides* with concentrations of 5 ppm). At 4 and 8 h, the highest values of cortisol on K (+) treatment K, whereas value of cortisol on A and B treatments different significantly. Finally, observation of post transportation from cortisol values on all the treatments did not different siginificantly. Cortisol levels at a time when the fish has not been treated range 86.73 – 92.15 ρg/mL whereas cortisol levels during transport between 90.52 – 401.3 ρg/mL (Table 10 & Figure 6).). Levels of cortisol a fish that has the highest value will be transported at the treatment clove oil followed by granting preferential treatment without giving the oil *A. conyzoides* (control). On treatment of the granting of leaves of *A. conyzoides* essential oil remains lower. The levels of cortisol in fish of rainbow trout that are experiencing acute stress is the average 45.16 µg/dL whereas in fish that are not experiencing stress is lower, i.e. an average of 31.50 µg/dL (Kubilay & Ulukoy, 2002) (Table 9.). There is a difference between the levels of cortisol on this research with the research conducted by Kubilay & Ulukoy (2002) because according to Iwama *et al.* (2006) to avoid over or sub estimation cortisol response please note the level of the basal cortisol in fish testing because it's very specific and individual stresor given also very specific. In this research reported that an increase in starting observation 0.5 h to 1 h and cortisol levels after 8 h of transportation showed a decrease in all treatments. According to Iwama *et al.* (2006) fish are generally achieving the highest concentration of cortisol at 1 h after experiencing stress and back to the basal levels after 6 h.

Interestingly, at 0.5 h the highest tachiventilation values contained on the B treatment (essential oils of *A. conyzoides* with concentrations of 10 ppm) different significantly with A treatment (essential oils of *A. conyzoides* with concentrations of 5 ppm) (Table 10 & Figure 6.). At 8 h, the highest tachiventilation on K (+) treatment (clove essential oil with concentrations of 5 ppm). Observation of post transportation showed that the highest tachiventilation on K (-) treatment (control, without essential oil). Tachiventilation is one of the external sign of fish in stressful conditions. Rapid movement in tachiventilasi is the effect of hormonal release Catecholamines during fish experience stress (Ross & Ross, 2008). Tachiventilation quick movements pointed operculum in fish. The value of tachiventilation before the treatment of transport ranged from 38 - 40 bit/3min while after treatment ranging from 12 – 132 bit/3 min (Table 9). Improvement of tachiventilation occurred at the beginning of transportation until 1 h, and then it decreased to 8 h. The tachiventilation activity is influenced by catecholamines hormone produced by kromafin cells in the anterior portion of the kidney. Catecholamines hormone have a role in regulating cardiovascular function and respiration (Barton, 2002). Hormone release catecholamines during stressful conditions take place instantly. Reaction evoked occurs within seconds to minutes (fight or flight reaction) for a few minutes to a few hours (Ross & Ross, 2008). Increased movement of the operculum is also used to increase oxygen consumption of fish used to swim, the movement of defence and migration (Gibson & Mathis, 2006). Sensitive indicators in fish physiology of stress response are tachiventilation. Tachiventilation function to measure the level of stress on the fish. Tachiventilation is a sympathetic response in response to the rapid response by stresor. Tachiventilation related to metabolic rate. Response to stress can cause a catabolic response and modulating metabolism, thus causing the occurrence of variation on tachiventilation (Barreto & Volpato, 2011). The addition of essential oils of *A. conyzoides* leaves in transport media provides value tachiventilation be lower than without the provision of essential oils. Low tachiventilation values during transport on granting *A. conyzoides* leaves essential oil treatment due to the influence of *A. conyzoides* leaves essential oil as analgesic activity. Tantarpale *et al*., 2012 showed that tachiventilation also influenced variety of stimuli such as changes in the environment (water temperature, pH, oxygen levels and residual molecules. A. conyzoides leaves essential oil has a sharp smell and has been fully tested as an analgesic, anti-inflammasi and anti-pyretic (Kamboj & Saluja, 2008).

The highest glucose values are found on the B treatment that it was different significantly with other treatments. At 8 h, the highest blood glucose values found on the B treatment, however it was different significantly with the A and K (+) treatments (Table 10 & Figure 6). Glucose levels in this research (before the treatment of transport) are ranging from 99-101 mg/dL, whereas during transportation on 8 h range 63-160 mg/dL (Table 9). Normal glucose levels in the cyprinid, is 40-90 mg/dL even reached 110 mg/dL (Patriche,

2009). On the Carp exposed to hypoxia, stress density and the breeding reportedly can increase blood glucose than 5 mmol/L to 10 mmol/L (Dobsikova *et al*., 2006). Glucose levels in the blood will be easy to change under the influence of internal and external factors. The concentration of glucose in the blood can be kept within the limits of normal through homeostatis mechanism. Porchas *et al.* (2009) reported that factors that influence the response of glucose are nutrition status, species and stage of development, stress hormones such as catecholamines, cortisol and various things that are affected by internal and external conditions on fish (anoxia, nutritive stress, pollution, and physical stress). Cortisol leads the process of gluconeogenesis and glycogenolysis on the fish but it can also lead to increase releasing of catecholamines from the chromafin cells that ultimately improving the glycogenolysis, modulation of cardiovascular and respiratory functions (Reid *et al*., 1998). The increase in blood glucose levels are one form of response of fish to the stressful conditions. Stress is a process that requires energy, glucose as an energy source will be used to adjust to stressfull conditions (Iwama *et al*., 2006). Levels of glucose in the treatment of essential oils have lower values than all the treatment this is due to the chemical content of essential oils contained in the form of precocene II has the ability to lower glucose levels in blood (Nyunai *et al*., 2010). Adebayo *et al*. (2010) reported that precocene II was probably the active phytochemicals that are responsible for the activity of hypoglycemic at this plant.

The highest value of Na^+ was found on B treatment at 0.5 h. However, observation of Cl^- at 0.5 h showed that the highest value found on the K (+) treatment (Table 10 & Figure 6). The result of Cl^- at 8 h showed that the highest value of Cl^- was found on B treatment. Levels of Na^+ and Cl^- ions before transported range 80 mmol/L (Na^+) and 100 mmol/L (Cl^-). According to Stoskopf (1993), the basic values of the electrolyte from Na^+ and Cl^- varies with species and the environment, ranging from 150 mmol/L and 130 mmol/L of Cl^-, respectively. Decreased body ions showed a stress on the fish. The Na^+ ion ranges between 116.33 - 177 mmol/L while the Cl^- ion range between 92 – 114 mmol/L (Table 9). Catecholamines and cortisol increase over the arrest and transport (Perry & Bernier, 1999). Adrenaline causes several physiological responses to acute stress and improve blood pressure that triggers the loss of NaCl through gills. On the salmon, the fish will die after the loss of plasma NaCl 35% as a consequence of handling an acute (Inoue & Moraes, 2006) (Table 9).

Based on the analysis of the MARS program were obtained the equations below Y = 15.538 + 29.415 * BF1-12.163 * BF3 - 4.859 * BF4. (Y = mortality; BF1 = maximum time; BF3 = maximum concentration; BF4 = density of fish). It means that the time of transport by administering essential oils *A. conyzoides* on 1 ppm with the amount of fish more than 3 tails in 0,3 liter of water will be able to live if it will be transported for more than 5 h. Analysis of MARS also pointed out that time and concentration of fish are more determining compared fish density when it was transported (Table 13). The value of survival rate from koi fish are transported for 8 h with a variety of treatment gives results that are no different for it continued with the treatment concentration on a range of density. Due to the *A. conyzoides* leaves essential oil has a good response to the fish physiological and more efficiently in fish transportation.

Stress reducing substances aimed at reducing stress, reduce fish activity and decreases metabolism. Many of anaesthetic and sedative or analgesic drugs used in vertebrate animals to reduce stress on fish (Neiffer & Stamper, 2009). On the biological assay activity, essential oils are dissolved into the water, giving the *A. conyzoides* leaves essential oil dissolved, continuously will pass through the gills. Lamella's gills are designed for gas exchange with the surface area of a wide and thin with vascular epithelium in the middle section of the capillary cells. According to Ross & Ross (2008) a technique used for anesthesia in solution depends on the type of drug anestesinya, the solution will be ingested by fish and molecular medicine will be diffused rapidly into the blood vessels in the secondary lamella to the efferent pathways of arteries, it is the fastest route to the central nervous system. Component of the aroma of essential oils quickly interact when inhaled, these compounds interact with central nervous system and directly stimulate the olfactory system, and then this system will stimulate the brain's nervous equilibrium below the cerebral cortex (Buckle, 1999). Fragrant compounds or fragrance of essential oils from a plant material has been proven also can affect the locomotor activity (Buchbauer *et al*., 1991). Locomotor activity is an activity of the motion as a result of changes in the electrical activity caused by changes in the permeability of the membrane postynaptic and by the release of transmitter by presynaptic neurons in the central nervous system (Gilman *et al*., 1991). This study, using clove oil as control it because in the field of fisheries, the clove oil contains eugenol is an alternative material that anesthesia can be used on many different types of fish. Ross & Ross (2008) suggests that the clove oil has an effectiveness such as MS-222 for anesthesia on rainbow trout juvenil and mature and does not affect the swimming speed of post anesthesia. According to Zahl *et al.* (2012) eugenol has hampered on sodium, potassium and calcium voltage gated channel. Li *et al*. (2007) stated the flow of electric current in the Na^+, K^+, Ca^{2+} volted gated channel plays an important role in shaping high-low phase of action potential. While K^+ volted gated channel is another group of membrane proteins that play a role in determining the basic nature of the electricity from the excitable cell, is also very important for the transportation of information and potential action by the terminal processes, repolarization neurons and the regulation of the release of neurotransmitters from a presynaptic terminal. The primary function of the nerve terminals of the transmitter presynaptik is releasing and also enables the target

cells of the post synaptic. Nearly all the steps to remove the transmitter always involves ion channel (Meir *et al*., 1999). According to Ikawati (2008) ion channel is a membrane protein which is found in the cell membranes and the lipid layer of several sub units composed of proteins forming porous. The opening and closing of ion channels governed by a chemical compound, an electrical signal, or mechanical force, depending on the type of the used channel. By regulating and controlling the flow of ions, ion channel can keep the negative charge possessed by cells on the conditions of the break. Voltage activated ion channels (voltage-gated channels) is an ion channel responses against the transmembrane potential of any changes. The canal will open in response to the occurrence of depolarization, and will close the case of hyperpolarization. An example is the ion Na^+ and K^+ channels in nerve cells and muscles, and canals that control the release of neurotransmitter Ca^{2+} on nerve endings presynaptic. Inhibition of current flow on K^+, Ca^{2+} and Na^+ volted gated channel is an analgesic effect on contribution to eugenol (Zahl *et al*., 2012).

Bandotan plant is a plant that is a central analgesic and essential oils of *A. conyzoides* promising narcotics analgesic agents as an activity through the opiodergic receptors (*Almeida et al*., 2001). Kamboj and Saluja (2008) reported that *A. conyzoides* essential oils influential in the activity as an analgesic and antipyretic on mice and rat. Content of terpenes and other chemical groups on essential oil has a molecular structure that is simple and has a molecular weight components are lightweight and high solubility in fats. These components can be inserted on blood brain barrier and acts on the central nervous system (de Sousa, 2011). Blood brain barrier is related to the size of the molecule and is directly related to solvency in the fat. Gas and water can be through the blood brain barrier quickly but glucose and electrolytes is slower. Blood brain barrier are not permeable to proteins, plasma and large molecules. According to Ross & Ross (2008) analgesia is an effort to escape from the pain. It will be block pain perception with no or by holding the ability of other sensors or motor function control is continued. In animals is reflected by a reduction in the response to noxious stimuli. In fish, the subject looks when analgesia it has not given a response or simply marked with the normal response from the noxious stimulus. Physical and motor response alerts to be indicators of analgesics. Pain receptors (nosiception) on a fish similar to other vertebrates, there is at last the spinal nerves (the tail) and nerve fibers in the trigeminal (head) and the entire surface of the body including the fins (Chervova & Laphsin, 2000). Information from nociception derived from neurons in the spinal cord or trigeminal caudal nucleus of brain stem. Then the response distributed to central nervous system to support the formation of behavior. In fish, the response behavior to withstand the pain stimulus with the move to leave the source of the pain. Essential oils of *A. conyzoides* promising analgesic narcotics agents as its activity through the opiodergic receptor. Narcotic analgesics according to Syamsudin & Darmono (2011) is a drug that works on the central nervous. The analgesic medications include drugs with central nervous system mechanisms depreciate either in part or whole, or work specifically. Opioid ingredients usually resembles a three-work family peptides in the brain known as endorphins, enkefalin and dinorfin. The peptide is opioid agonist. This peptides along with some non opioid peptides (ACTH, MSH and lipoprotein) solved from prekusor POMC protein (Olson, 2004). At a time when stress or pain endogenous peptide receptor works on opiates. According to Sneddon (2012) opioid drugs are exemplified by morphine produces an analgesic with 3 opioid receptors on their role i.e. μ, δ, and κ located on the cell membrane of neurons. Presynaptic action on opioid neurotransmitter release is inhibited, not only to block the activity of nociceptor but also block the transmission center. The common carp, μ, δ, and κ receptors found in the hypothalamus, pars distalis, pars intermedia pituitary, kidney, thymus, the anterior part of the spleen and leukocytes (Bernier *et al*., 2009). According to Beale & Black (2010) all opioid receptor is a G-protein receptor including G protein-coupled receptor and arranged in 7 transmembrane domain. When the receptors are activated then part of G-proteins will diffuse in the membrane and cause inhibition of the Adenilate Cyclase. Decreased enzyme activities in line with the decrease in the formation of C-AMP (Cyclic Adenosine Monophospat) that regulate cellular processes. One of inhibitory processes that enabled the Volted Gated Calcium Channel Influx on C-fiber nociception and finally subjected to nerve cells in hyperpolarization and lower the firing and release the neurotransmitter glutamate such pain and substance P. Agonis μ receptor is producing analgesia, emphasis on respiration, decrease of gastrointestinal motility, euphoria, consumption and the release of hormones. According to Ikawati (2008), based on his inhibiting enzymes that work then the adenil siklase is a protein Gi (Inhibitory G Proteins). At the time of stress, the endogenous peptide on opioid receptor will work (Olson, 2004) are expected with the properties of the essential oils of *A. conyzoides* leaves as analgesic opioids. Then fish stress (caused by transportation) will bind to opioid receptors in the membrane of fish neurons that influence of stressor is perceived by the organism can be reduced. Finally, the reduced influence of stressor is expected to affect the response of primary, secondary and tertiary from fish.

4. Conclusion

The essential oil of *A. conyzoides* leaves as a potentially for reducing stress reducing substance related koi fish transportation (*C. carpio*) because it has an influence upon response stress that is, a decrease in blood plasma, levels of cortisol, tachiventilation, blood glucose, increased of Na^+ and Cl^- in blood plasma and not occurring

death during transportation.

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Table 1. Chemical Composition Analysis Results of Clove Essential Oil.

No	Component	Contain (%)
	Fenilpropanoid	
1	Eugenol	71.901
2	Naphtalene	0.379
	Terpen	
1	Caryophyllene	23.219
2	α- Caryophyllene	3.2
3	Caryophyllene oxide	1.079

Table 2. Chemical Composition Analysis Results of A. *conyzoides* Essential Oil.

No	Komponen	Kandungan (%)
	Chromen	
1	Ageratochromene (Precocene II)	62. 381
2	7-Metoxy-2. 2-dimethylchromene (Precocene I)	15. 965
3	6-Vinyl-7methoxy-2.2-Dimethyl chromene	1.57
	Terpen	
1	Caryophyllene	7.185
2	β-Sesquiphellandrene	2.086
3	Germacrene D	1.04
4	α-Caryophyllene	1.155
5	Epi- bicyclosesquiphellandrene	0.486
6	1H-Cyclopropa [A] Napthalene	0.491
7	Caryophyllene oxide	0.826
8	Bicyclogermacrene	0.446
9	3.3.4.5.7-Pentamethyl-1-Indanone	0.728
10	α-Farnesene	0.279
11	Phenantrene	0.18
12	β-Cubebene	0.24
	Kumarin	
1	2H-1-Benzopyran-2-one	2.074
	Fenol	
1	2-Methoxy-4-vinyl phenol	1.375

Table 3. Analysis Result of Essential Oils Solubility in Water

Amount of Bandotan's leaves essential oil	Water solubility	Amount of Clove essential oil	Water solubility
1 µL	-	1 µL	-
0.9 µL	-	0.9 µL	-
0.8 µL	-	0.8 µL	-
0.7 µL	-	0.7 µL	-
0.6 µL	-	0.6 µL	-
0.5 µL	+	0.5 µL	+
0.4 µL	+	0.4 µL	+
0.3 µL	+	0.3 µL	+
0.2 µL	+	0.2 µL	+

Information : - = insoluble
+ = soluble

Table 4. Average of Koi Carp Mortality During Biological Activity Test.

Concentration (ppm)	A. *conyzoides* Essential oil Mortality (%) ± SD	Clove essential oil Mortality (%) ± SD
0	0±0	0 ±0
10	0±0	0 ±0
20	13.33±2.31	13.3 ±2.31
30	20±3.46	13.3 ±2.31
40	53.33±1.15	100 ±0
50	100±0	100 ±0
60	100±0	100 ±0
70	100±0	100 ±0
80	100±0	100 ±0
90	100±0	100 ±0

Table 5. Range of Water Quality During Biological Activity Test.

Parameter	Range	Reference
Dissolved Oxygen (ppm)	4 – 6	4-10 ppm (Stoskopf, 1993)
Ammonia (ppm)	0.006-0.27	< 0.2 ppm (Stoskopf, 1993)
Water Temperature (°C)	28-31	3-35°C (Stoskopf, 1993)
pH	7.5-8.4	6.5- 9 (FAO, 2011)

Table 6. Average Mortality Rate of Koi Carp During Transportation

A. *conyzoides* essential oil		Clove essential oil	
Concentration (ppm)	Mortality (%) ± SD	Concentration (ppm)	Mortality (%) ±SD
0	0 ±0	0	0 ±0
5	0 ±0	5	0 ±0
10	0 ±0	10	10 ±1.73
15	20 ±1	15	10 ±1.73
20	40 ±1	20	10 ±1
25	50 ±2	25	13.3 ±0.58

Table 7. Average Tachyventilation (bit/3 minute) and Blood Glucose Level (mg/dl) of Koi Carp During Transportation.

Tachyventilation (bit/3 minute)									
Concentration (ppm)	Observation Hours								
	0	1	2	3	4	5	6	7	8
0	128.67	129.33	127.67	103.33	65.67	61.33	60.67	50.33	42.67
5	125	61.33	59.67	56.33	49.33	45.67	34	29.67	21.33
10	127.67	92.33	87.33	84.67	75	73	66.67	59.67	53
15	127	112	109.67	96.33	93	76	60.67	39.33	39.67
20	130.33	94.33	94.33	82	81.67	70.67	64.33	59.33	44
25	129.33	88.33	54.33	38	46.67	50.33	52.33	47.33	48.67

Blood Glucose (mg/dl)					
Concentration (ppm)	Observation Hours				
	0	2	4	6	8
0	26.33	27.33	64.33	66	71.67
5	27	38	65	52.67	46.67
10	26.67	189.67	170.67	230.67	217
15	27	154.33	229.33	197.667	200.33
20	26.33	154	250.33	209	221.33
25	26.67	243.33	302.67	302	299

Table 8. Range of Water Quality During Transportation.

Water Quality Parameter	Observation time (hours)			Reference
	0	4	8	
Water Temperature (ºC)	26-27	21-22	21-22	3-35°C (Stoskopf, 1993)
pH	8.9-9	7.6-7.8	7.7-7.8	6.5- 9 (FAO. 2011)
Dissolved Oxygen (ppm)	6	6	6	4-10 ppm (Stoskopf, 1993)
Amonia (ppm)	0.02	0.09-0.18	0.18-0.27	< 0.2 ppm (Stoskopf. 1993)

Table 9. Range of Cortisol. Blood Glucose. Tachyventilation. Na^+. and Cl^- Levels in Normal Condition.

Parameter	Result	Reference
Cortisol (ρg/ml)	86.73 – 92.15	31.50 µg/dl. (Kubilay and Ulukoy. 2002).
Tachyventilasi (bit/3 menit)	38 - 40	
Blood Glucose (mg/dl)	99 - 101	111 (Stoskopf. 1993)
Ion Na^+ (mmol/L)	82	150 (Stoskopf. 1993)
Ion Cl^- (mmol/L)	100	130 (Stoskopf. 1993)

Table 11. Average Survival Rate of Koi Carp During Transportation.

Treatment	Survival Rate (%)
K(-)	100^a
A	100^a
B	100^a
K(+)	100^a

Index : Different superscripts in one coloum shows a significance difference ($p<0.05$). **K(-)** (Control /without treatment of A. *conyzoides*'s essential oils). **A** (Treatment with 5 ppm of A. *conyzoides*'s essential oils). **B** (Treatment with 10 ppm of A. *conyzoides*'s essential oils). and **K(+)** (Treatment with 5 ppm of clove essential oil).

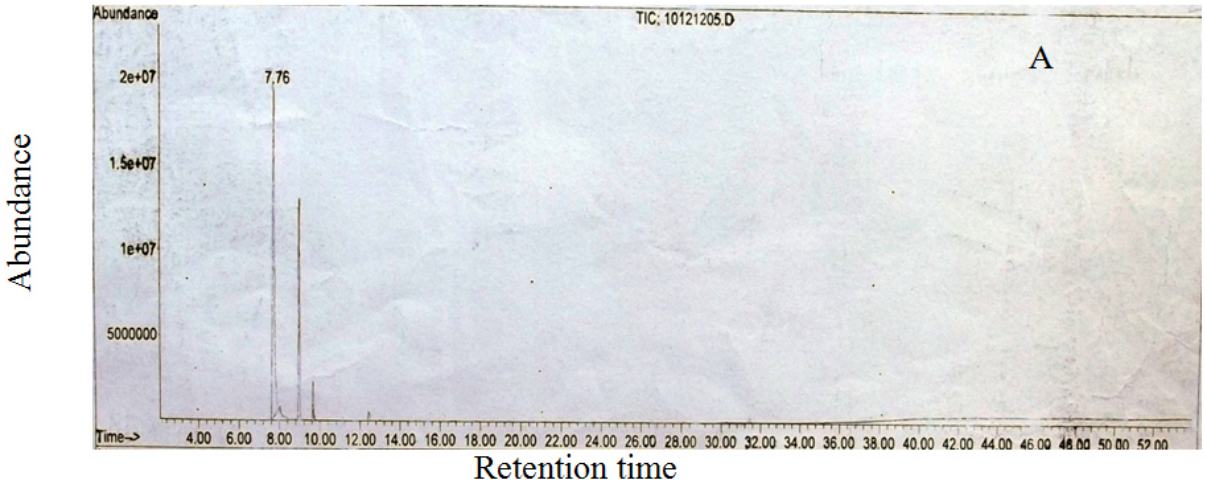
Table 12. Average Range of Water Quality During Transportation

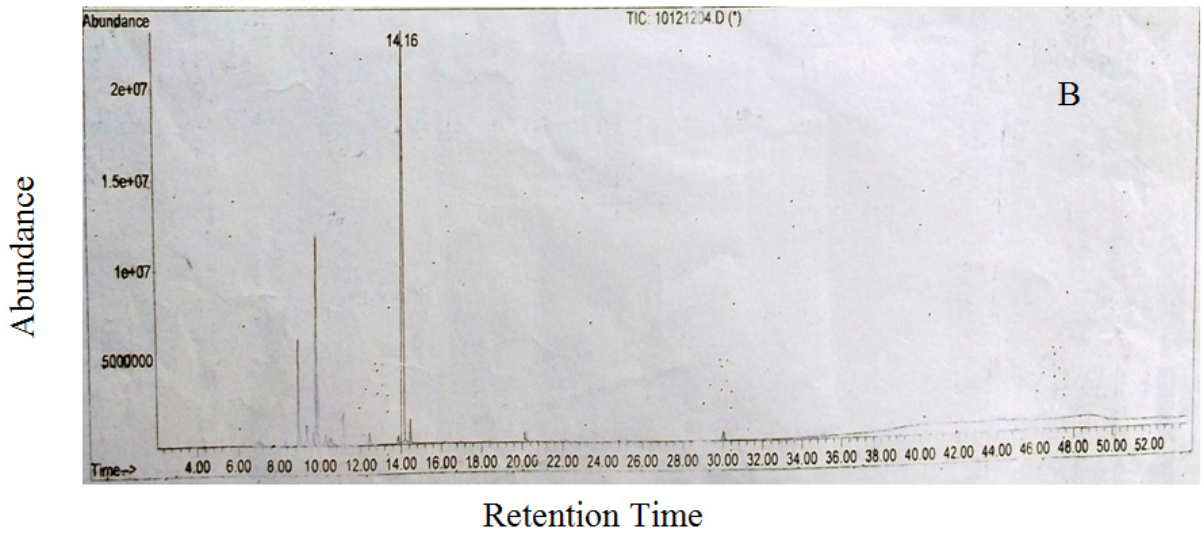
Observation Time (hour)	Concentration (ppm)	Water Quality Parameters			
		Temp. (ºC)	DO (ppm)	pH	NH3 (mg/l)
0	0	28	6	8.9	0.05
	5	27	6	8.7	0.05
	10	28	6	8.9	0.05
	C5	27	6	8.8	0.05
4	0	21	6	7.7	0.18
	5	20	6	7.7	0.09
	10	21	6	7.8	0.09
	C5	21	6	7.7	0.09
8	0	20	6	7.8	0.27
	5	21	6	7.7	0.16
	10	20	6	7.7	0.18
	C5	20	6	7.8	0.18
Reference		3-35°C (Stoskopf, 1993)	4-10 ppm (Stoskopf, 1993)	6.5- 9 (FAO. 2011)	< 0.2 ppm (Stoskopf. 1993)

Table 13. Average Mortalities (%) in Various Densities

Concentration (ppm)	Densities (fish)	Observation Time (Hour)							Total
		0	4	8	12	16	20	24	
0	1	0	0	0	0	0	0	0	0
	5	0	6.67	0	13.33	0	0	13.3	33.27
	10	0	6.67	13.3	0	0	23.3	43.3	86.57
	20	0	5	6.67	8.33	15	11.67	40	86.67
5	1	0	0	0	0	0	0	0	0
	5	0	0	6.6	0	13.3	0	0	19.93
	10	0	6.67	0	13.33	16.67	20	0	56.67
	20	0	3.33	8.33	8.33	10	11.67	16.6[a]	58.33
10	1	0	0	0	0	0	0	0	0
	5	0	0	6.67	20	6.67	26.67	6.67	66.68
	10	0	13.33	10	10	16.67	6.67	16.67	73.34
	20	0	8.33	10	16.67	8.33	18.33	16.67	73.34

Index : Different superscripts in one coloum shows a significant difference ($p<0.05$).





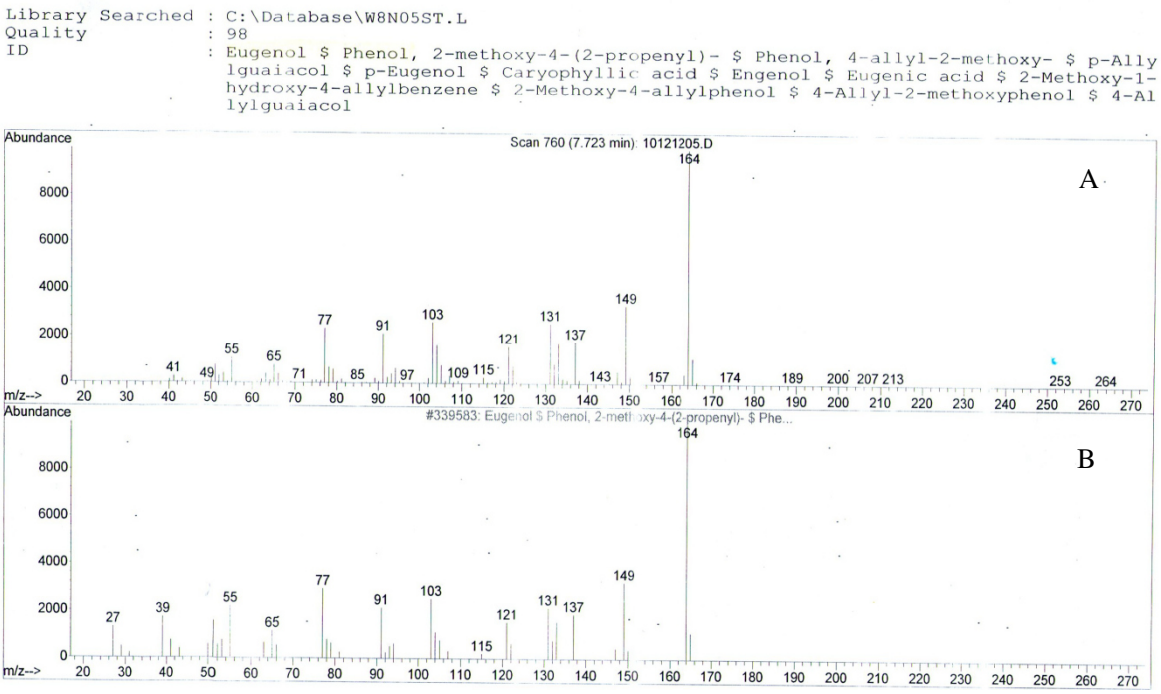


Figure 2. The Chromatogram Results of Eugenol in Clove Essential Oil Sample (A) and GC-MS Library (B)

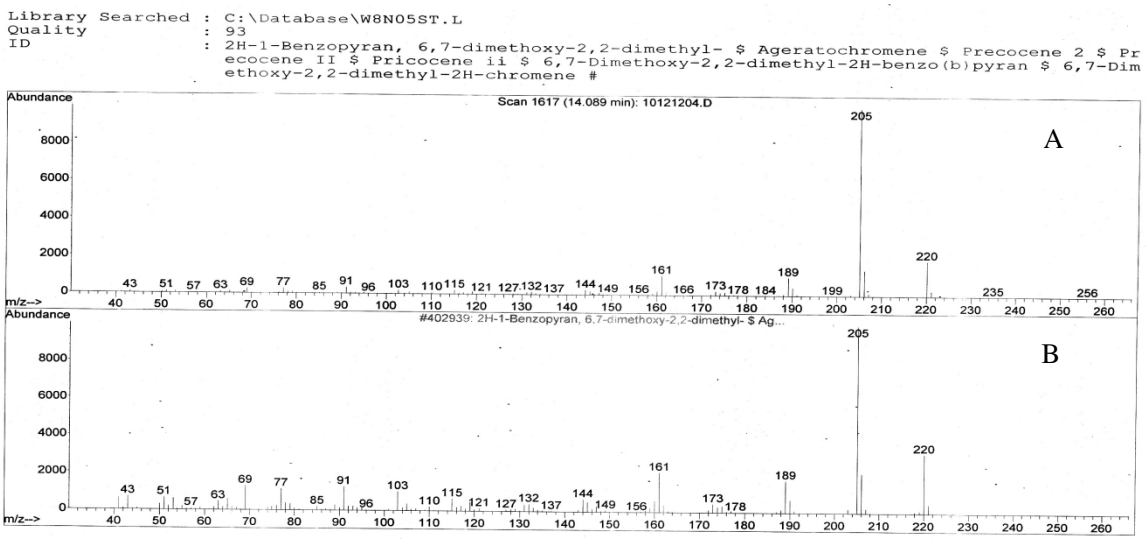


Figure 3. The Chromatogram Results of *Ageratochromene* (Precocene II) in A. *Conyzoides* Essential Oil Sample (A) and in GC-MS Library (B).

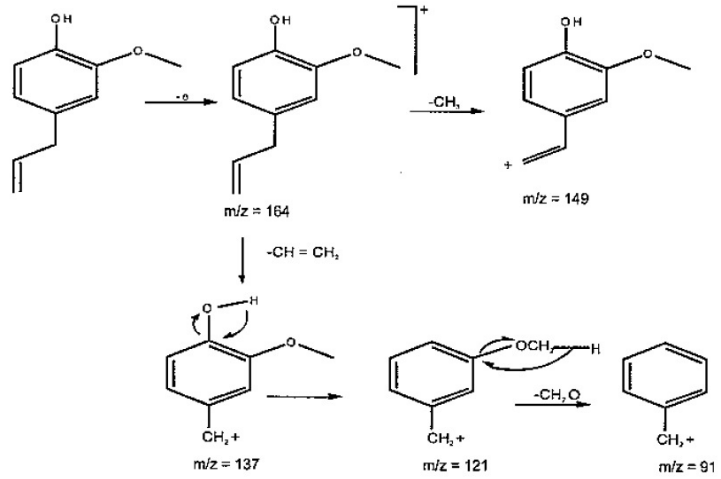


Figure 4. Fragmentation Pattern of Eugenol in Mass Spectrometry (www.scribd.com).

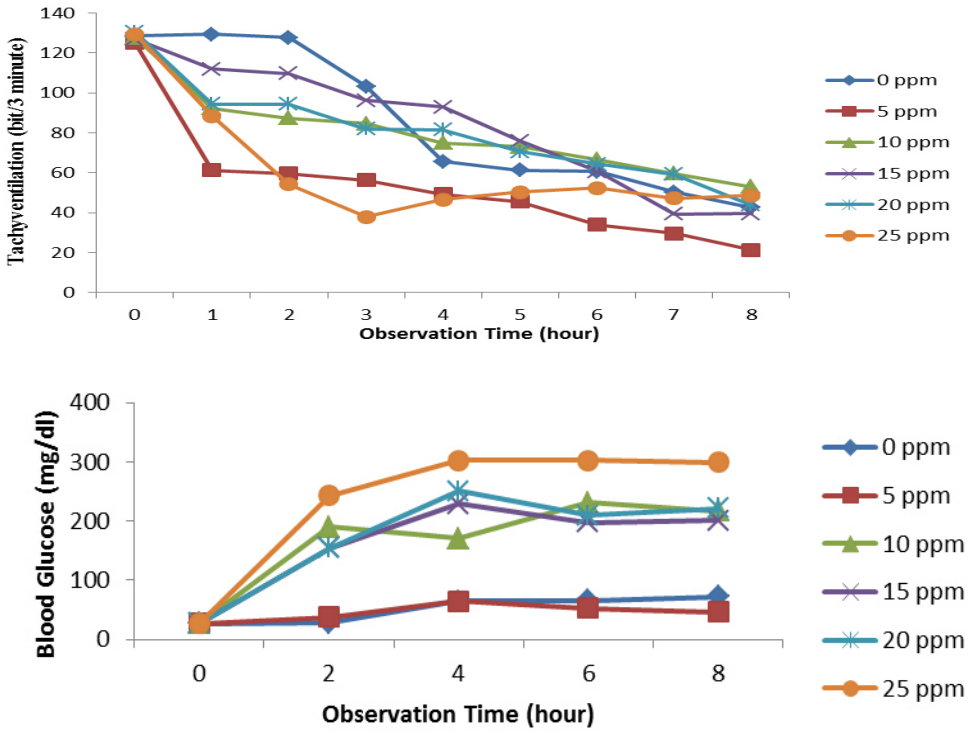


Figure 5. Graphics of Average Tachyventilation (bit/3 minute) and Blood Glucose Levels (mg/dl) of Koi Carp During Transportation.

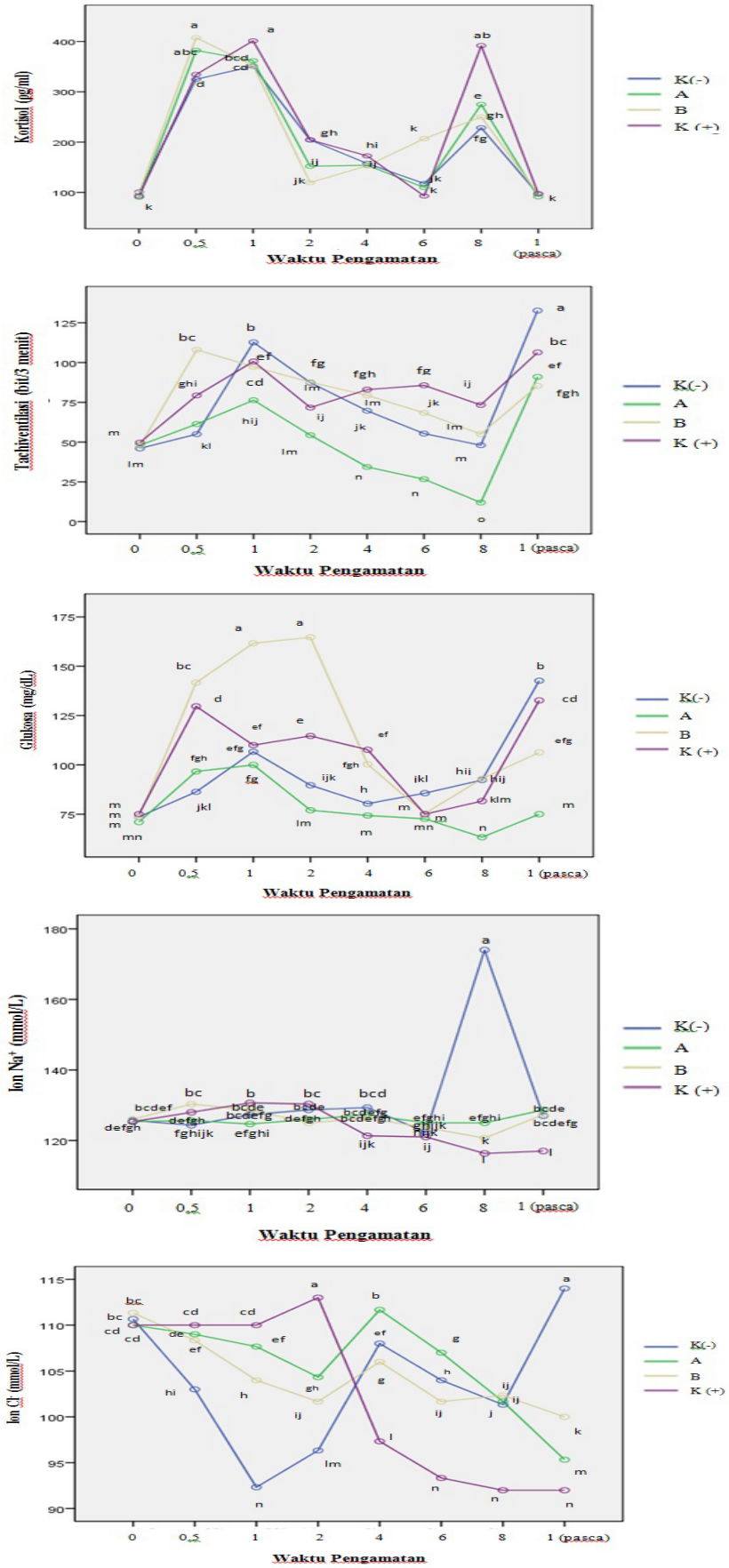
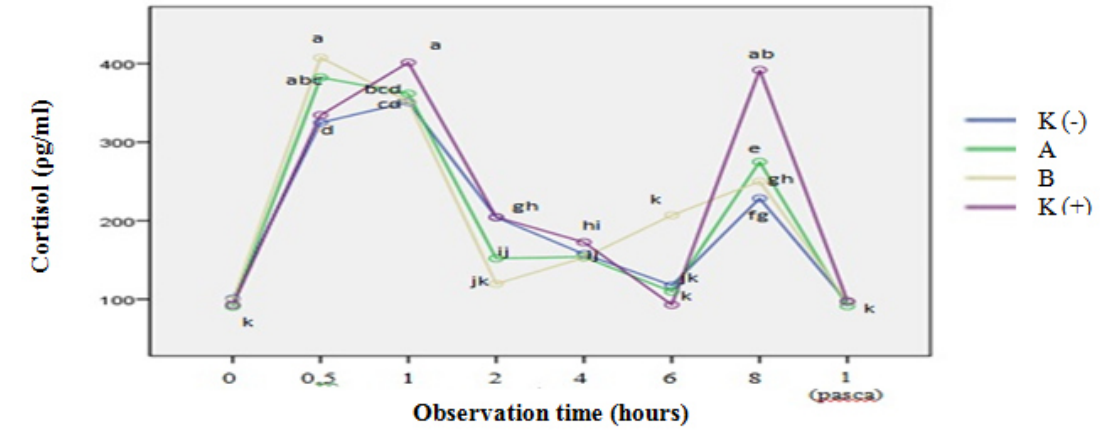


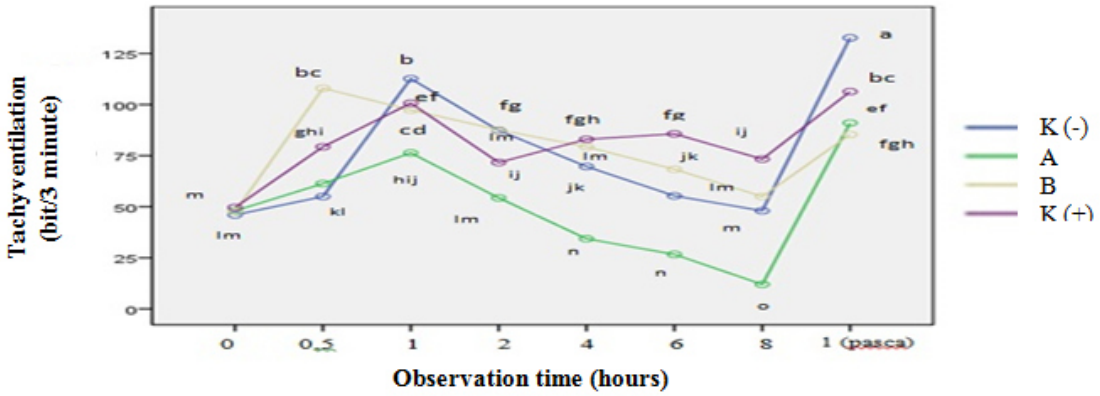
Figure 6. Graphics of average Cortisol. Tachyventilation. Blood Glucose. Na^{+}. and Cl^{-} levels during experiment.

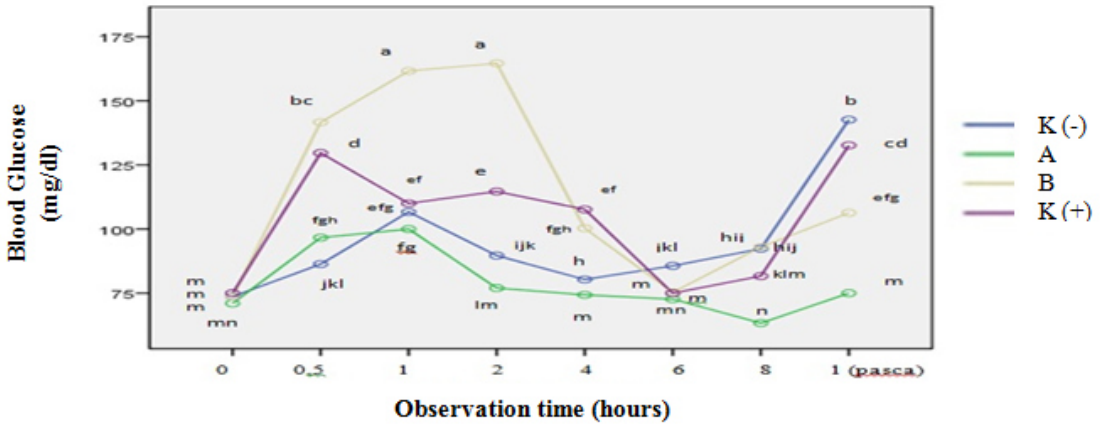
Table 10. Average of Stress responses

Cortisol (ρg/ml)								
	Observation time (hour)							
Concentration (ppm)	0	0.5	1	2	4	6	8	1 (pasca)
K(-)	100.71	324.84	350.98	204.25	157.51	117.65	228.43	96.33
A	90.52	382.35	361.77	151.96	153.92	109.81	274.84	91.23
B	98.81	407.52	351.96	119.60	152.94	206.90	250.00	96.84
K(+)	92.81	334.31	401.30	204.57	172.55	92.92	391.80	97.55
Tachyventilation (bit/3 minute)								
Concentration (ppm)	Observation time (hour)							
	0	0.5	1	2	4	6	8	1 (Pasca)
K-	46	55	112.67	87.33	69. 67	55. 33	48	132.67
A	48	61.33	76.33	54. 33	34. 33	26. 67	12	91
B	47.67	108	97. 33	87. 67	79. 33	68. 33	55	85.33
K+	49. 67	79. 33	100. 67	71. 67	83	85. 67	73. 33	106.33
Blood Glucose (mg/dl)								
Concentration (ppm)	Observation time (hour)							
	0	0.5	1	2	4	6	8	1 (Pasca)
K-	73. 67	86. 33	106. 67	89. 67	80. 33	85. 67	92.33	142.67
A	71	96.67	100	77	74. 33	72. 67	63. 33	75
B	73. 67	141. 67	161.67	164.67	100.33	75.33	93	106.33
K+	75	129.67	110	114.67	107.67	75	81.67	132.67
Na^+ (mmol/L)								
Concentration (ppm)	Observation time (hour)							
	0	0.5	1	2	4	6	8	1 (Pasca)
K-	125.67	124.33	127.33	128.67	129.33	122.33	174.00	127.00
A	125.33	125.67	124.67	126.00	127.33	125.00[b]	125.00	128.67
B	126.00	130.33	128.67	125.00	126.33	123.67	120.67	127.33
K+	125.33	128.00	130.67	130.33	121.33	121.00	116.33	122.00
Cl^- (mmol/L)								
Concentration (ppm)	Observation time (hour)							
	0	0.5	1	2	4	6	8	1 (Pasca)
K(-)	110.67	103.00	92.33	96.33	108.00	104.00	101.33	114.00
A	110.00	109.00	107.67	104.33	111.67	107.00	101.67	95.33
B	111.33	108.33	104.00	101.67	106.00	101.67	102.33	100.00
K(+)	110.00	110.00	110.00	113.00	97.33	93.33	92.00	92.00

Notes: **K(-)** (Control /without treatment of A. *conyzoides*'s essential oils). **A** (Treatment with 5 ppm of A. *conyzoides*'s essential oils). **B** (Treatment with 10 ppm of A. *conyzoides*'s essential oils). and **K(+)** (Treatment with 5 ppm of clove oil).


Cortisol (pg/ml)
400
300
200
100
0
0,5
1
2
4
6
8
1 (pasca)
Observation time (hours)
K (-)
A
B
K (+)


Tachyventilation (bit/3 minute)
125
100
75
50
25
0
0
0,5
1
2
4
6
8
1 (pasca)
Observation time (hours)
K (-)
A
B
K (+)


Blood Glucose (mg/dl)
175
150
125
100
75
0
0,5
1
2
4
6
8
1 (pasca)
Observation time (hours)
K (-)
A
B
K (+)

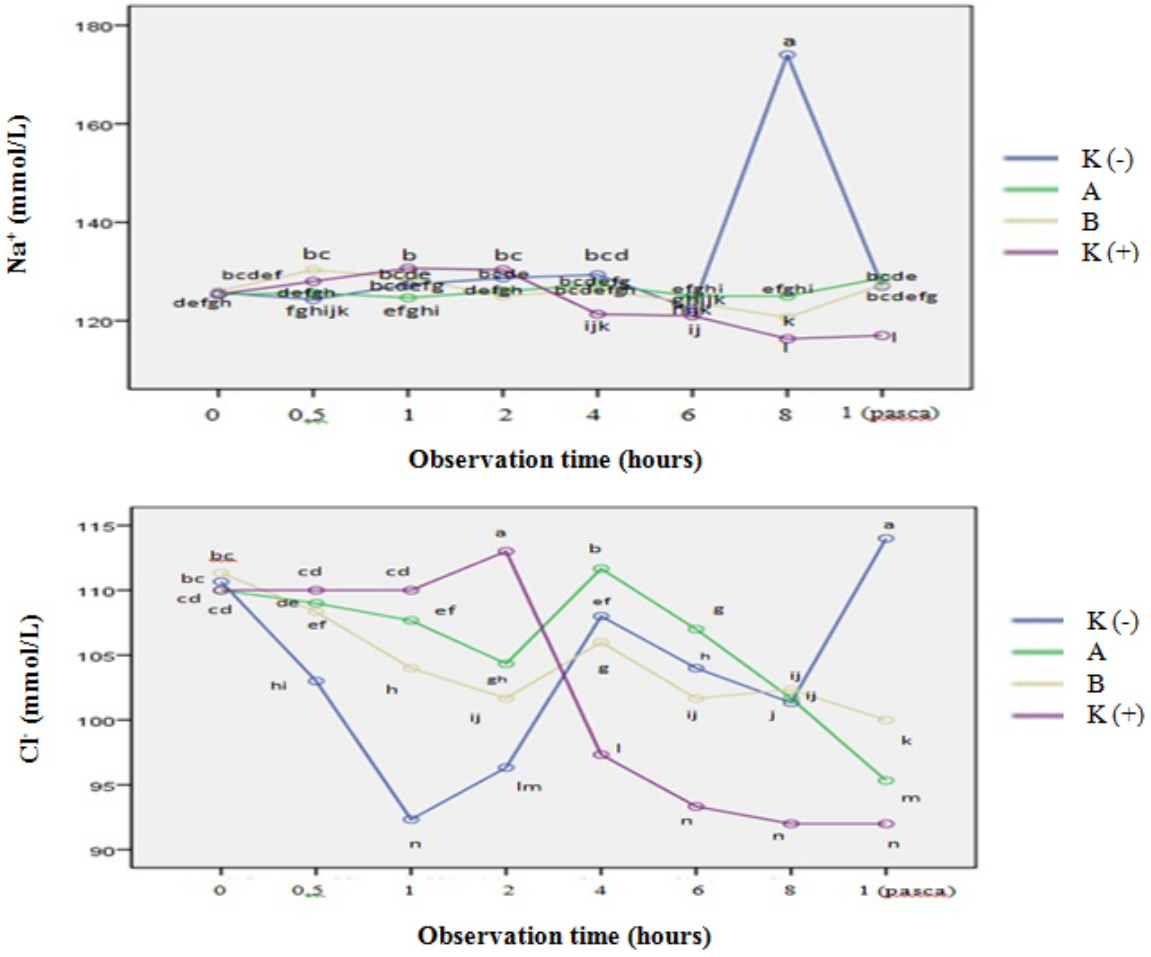


Figure 6. Graphics of average Cortisol. Tachyventilation. Blood Glucose. Na^+. and Cl^- levels during experiment.

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