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Cardio-toxicity and oxidative stress induced by Egyptian Cyanobacteria *Oscillatoria brevis*, toxicity and mechanism of action, an *in vitro* study

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Abstract: *Cyanobacteria or blue green algae have become more world wide spread, Oscillatoria brevis (O. brevis) is one of the most toxic species collected from freshwater Suez canal during algal bloom condition and was grown in blue green medium (BG-11) under laboratory conditions. In vitro assessment of cardio-toxicity of O. brevis was carried out by recording Electrocardiogram (ECG) after direct perfusion of isolated toads' hearts with 5µg/ml of O. brevis extract. Moreover, the mechanism of action of O. brevis extract on myocardium was revealed by the pharmacological tests using muscarinic receptor blocker (atropine sulfate), propranolol hydrochloride and verapamil. Oxidative stress induced by O. brevis was estimated by incubation of mice blood with 1µg/ml of O. brevis at 37 ^{0}C for 2, 4, 6, 8 and 24 hours respectively to determine the level of lipid peroxidation and antioxidants activities. Perfusion of isolated toads' hearts with 5µg/ml O. brevis extract significantly decreased the heart rate with an increase in the conduction time (P-R interval). Also, a remarkable increase in the power of ventricular contraction was observed, It was suggested that O. brevis could act through Ca^{2+} channels where verapamil application abolished the increase in myocardial contractility noticed after 10 minutes of O. brevis extract application. Lipid peroxidation and blood catalase activity significantly increased while a pronounced decline of blood glutathione concentration and erythrocyte superoxide dismutase activity were noticed. Oxidative stress induced by O. brevis could underlying the cardiotoxicity which is represented by sinus arrhythmia, ectopic beats and heart infarction.*

Key word: *O. brevis, ECG, Heart rate, Ca^{2+} channels, oxidative stress.*

I. Introduction

Cyanobacteria or blue green algae have become more world wide spread (Paerl, 2007). Which inhabitants of fresh, brackish and marine, Over forty species of freshwater cyanophytes have been implicated in toxic blooms, Microcystis, Oscillatoria, Anabaena, Nostoc which are the most toxic species water (Whitton & Potts, 2000). Harmful produced toxins are secondary metabolites known as cyanotoxins, are classified according to their toxicity into neurotoxins, hepatotoxins and skin irritants (Carmichael, 1997). Cyanotoxins are toxic to mammals and aquatic invertebrates. Sheep were died following ingestion of cyanobacterial polluted water (Krienitz et al., 2003). Since this was published many others have shown that toxic cyanobacteria can also intoxicate mammals, waterfowl and fish. Humans are also found to be intoxicated by cyanobacteria, either due to accidental ingestion of water during, for example, aquatic sports or following ingestion of drinking water contaminated with toxic cyanobacteria (Gehringer, 2003). Some species of Oscillatoria are toxic to human and animals, and are known to produce both neurotoxins (anatoxin) and hepatotoxin (microcyctins) (Hisbergues et al., 2003). Anatoxins can block the transmission of signals from neuron to neuron and from neuron to muscle, where microcystins cause bleeding in the liver (observed in mice after i.p. injection with *Oscillatoria brevis* extract (El-Manawy & Amin, 2004a).

Intravenous and intra-cerebro-ventricular administration of anatoxin-a resulted in an increase in mean arterial pressure (MAP), in the conscious rat with concomitant bradycardia (Sirén and Giora, 2004 & Madigan et al., 2000). Moreover, Guzman and Solter (2002) reported that IP administration of MC-LR into young male rats resulted in a decrease in body weight concomitant with a significant increase of ALP, AST, ALT, and GGT activities and elevated levels of BUN and creatinine. Fitzgeorge et al, (1994) investigated the co-effect of anatoxin-a toxicity with microcystin-LR, the most common cyano-hepatotoxin. They found a significant synergistic effect between anatoxin-a and microcystin-LR.

Gomaa et al., 2000 indicated *Oscillatoria brevis* is a taxic cyanobacterium in Egypt. Amin (2001) reported acute neurotoxicity signs in mice after i.p injection with extract of *O. brevis* collected from Port Said freshwater canal during bloom condition. A bloom of *Microcystis aeurginosa* and Oscilltoria species have been

reported in Suez governorate in 2003, 2004 associated with the emergency obnoxious taste and odor in drinking water (El-Manawy & Amin, 2004a).

The current study was conducted to investigate the influence of *O. brevis* on the electrical activity of isolated heart and its potential to cause adverse health effects by induction of oxidative stress.

II. Material And Methods

Sample collection, culture and extraction of toxin

The phytoplankton samples were collected seasonally from freshwater Suez canal, Suez, Egypt, from five stations in the canal during a period of 12 months. Species of phytoplankton were identified using the inverted microscope (Prescott, 1978). Stock algal bloom was maintained in BG-11medium for growth and toxin production, at room temperature supplied by sterilized air (Carmichael, 1985). An HPLC system used 600E pump system was used for lyophilized filtrate purification steps.

Chemicals and solutions

Atropine sulphate was purchased from Memphis Co, Dorpharm and Chem, Ind. Cairo, Egypt. Where concentration of 4 μg/ml Ringer solution was used on heart preparations. Verapamil hydrochloride (40 mg), the calcium channel blocker was used on the heart with a dose of 5μg/ml of Ringer solution. It was purchased from ADWIC pharmaceutical division, EL Nasr Pharm. Chem. Co., Abu Zabal, Egypt. Propranolol was bought from Sigma Co. and was used in experiments on the heart with a final concentration of 5µg/ml Ringer solution. The concentration of the blockers (atropine sulphate, verapamil and propranolol) used in the present work were applied according to Nabil et al. (1998).

Experimental animals

Adult male toads (*Bufo regularis*) were used as an experimental model for in vitro experiments. Also, male adult albino Mice weighting 25-33g were used in this study. Animals were housed under standardized conditions and allowed free access to food and water ad libitum and left for acclimatization before the start of the study.

Cardiac muscle experiments

Experiments on cardiac muscle were carried out on adult of isolated heart male toads (35–40 g each). Four groups of toads of ten animals of each group were used, the first group was directly perfused with 5µg/ml of *O. brevis* extract to study the electrical effects of *O. brevis* on the ECG. ECG data were recorded directly from the surface of the heart according to Nabil et al. (1998) before and after *O. brevis* application. ECG was taken before any application to serve as self control. After *O. brevis* perfusion, signals were recorded each 5 min for 30 min. Atropine sulphate, verapamil and propranolol, were applied after 10 min (the time in which the pronounced effect of *O. brevis* was recorded in all toxicological experiments) to reveal the mechanism of action on heart preparations (n = 5) .

Oxidative stress biomarkers

Mice were divided into 2 groups (10 mice /group) for biochemical study, the 1st group served as control where mice were slaughtered and blood samples were collected, EDTA (50μl) was added to all blood samples before incubation as an anticoagulant. Blood of the 1st group was incubated with 1ml of the vehicle (0.9% NaCl) to serve as control, while blood of the 2nd group was incubated with 1μg/ml of *O. brevis* at 37 ^{0}C for 2, 4, 6, 8 and 24 hour respectively. Samples of the whole blood were used for determination of glutathione and catalase according to the methods of (Beutler et al 1963 and Aebi, 1984), while superoxide dismutase (SOD) was measured in erythrocytes according to (Winterbourn et al., 1975). On the other hand, lipid peroxidation was estimated in serum of control and treated blood samples (Sharma and Wadhwa, 1983)

Statistical Analysis

The results were expressed as means ±S.E. Changes in the heart rate (HR) and the other ECG parameters of isolated toads' hearts after *O. bervis* extract or antagonist application were tested for the statistical significance using paired t-test (Snedecor, 1980) at $P<0.05$, $P<0.01$ and $P< 0.001$. Percentage of change (%) of electrocardiographic parameters of toads was expressed of the raw data before and after *O. bervis* extract application. Changes in the concentration of lipid peroxidation, GSH, catalase and SOD were tested using un-paired t-test to determine the difference of treatment from control group (Snedecor, 1980) at $P<0.05$, $P< 0.01$ and $P<0.001$.

III. Results

Electrophysiological results

In vitro experiments were accomplished to investigate the effect of the *O. brevis* extract on myocardium activity. ECGs were recorded from isolated toads' hearts before and after perfusion of *O. brevis*. Normal heart rate (HR) and different ECG parameters were measured from the recorded electrocardiograms of isolated hearts before any treatment to serve as self-control (0-time). The effects of direct perfusion of *O. brevis* on the HR, conduction time (P–R interval), depolarization and repolarization voltage (R and T wave) of the isolated hearts are summarized in Table1. Direct perfusion of isolated toad's heart with 0.5 μg/ml of *O. brevis* extract on the heart rate induced negative chronotropic effects (bradycardia) represented by a highly significant decrease of heart rate at different time intervals 10, 15, 20, 25, 30 min ($P< 0.001$). Bradycardia was accompanied with a significant negative dromotropic effect represented as an elongation of P-R interval of the recorded ECG at all the recorded times ($P< 0.001$). This indicates a decrease in the conduction velocity of impulses as shown in table 1 and Plate1.

A remarkable increase in the ventricular amplitude of contraction was noticed after direct perfusion of 0.5 μg/ml *O. brevis* extract on the isolated toad's heart indicated by a significant increase in the R-amplitude at different time intervals 10, 15, 20, 25, 30 min from toxin application ($P< 0.01$). Also, application of 0.5 μg/ml *O. brevis* extract on isolated toad's heart induced an increase in the ventricular repolarization voltage since a significant increase in the T-amplitude after different time intervals 10, 15, 20, 25, 30 min was noticed ($P< 0.01$) compared to self control as seen in table 1.

The percentage of change of the heart rate, P-R interval, depolarization voltage and repolarization voltage of the isolated toad's heart following *O. brevis* toxin perfusion within 30 minutes are represented in fig.1 A, B, C and D.

Furthermore, ECGs charts have shown examples of cardiac disorders induced by direct application of *O. brevis* extract on isolated toads' hearts (plate. 2) such as Wide QRS-Wave, Sinus arrhythmia, ST-depression and First degree block, Peaked P- wave, Ectopic beats, Negative QRS deflection, Second degree block and Complete heart block. Table 2 represents percentage of cardiac disorders incidents, as seen in the table 2, sinus abnormalities were the most frequent followed by different degree of atrioventricular blocker.

Mechanism of action of *O. brevis* extract on the cardiac muscle activity

In an attempt to reveal the mechanism of action of *O. brevis* extract on the cardiac muscle activity, 4µg/ml of atropine sulfate was used as muscarinic receptor antagonist, to ameliorate the decline in the heart rate and atrioventricular conduction velocity induced by *O. brevis* extract, post treatment with atropine sulfate did not abolish the negative chrono and dromotropic effect of *O. brevis* extract (plate3).

On the other hand, the β adrenergic bloker propranolol hydrochloride (5μg/ml) as well as verapamil (5μg/ml) as a selective antagonist L- type Ca^{2+} channels were used following perfusion of *O. brevis* extract, to suppress the pronounced increased of myocardial contractility. Propranolol could not abolish the positive inotropic effect, while verapamil application could inhibit the noticed increase in myocardial contractility after 10 minutes of *O. brevis* extract application. Fig.2. Plate 3 illustrates an action of *O. brevis* mechanism after atropine, propranolol and verapamil application on the isolated heart.

Oxidative stress biomarkers

Incubated of blood with *O. brevis* extract with a concentration of 1μg/ml for 2, 4, 6, 8, 24 hrs significantly increase plasma lipid peroxidation represented by an increase in thiobarbituric acid reactants (TBARS) level with percentage of change 88, 91, 91, 82, 81% compared to control group.

Furthermore, a pronounced decline of blood glutathione concentration was noticed after 2, 4, 6, 8 hrs of *O. brevis* extract incubation with percentage of change 7.3, 19.2, 12.2, 11% compared to control group. After 24hrs of incubation time, a remarkable increase was noticed with a percentage of change 9.9% compared to control group. In the mean time, the activity of blood catalase increased after 4 and 6 hours of incubation with 1μg/ml of *O. brevis* extract.

On the other hand, *O. brevis* extract significantly decreased erythrocytes superoxide dismutase activity with a percentage of change 82, 84, 106, 174, 155% compared with control group in all incubation times. The effect of *O. brevis* extract on plasma lipid peroxidation, blood glutathione, catalase and SOD activities are represented in fig.3.

IV. Discussion

In vitro experiments were carried out in the current study on the isolated perfused toad‘s heart, since isolated organ preparations offer several advantages over experimentation on the intact animals because they lend themselves to a definite evaluation of the role of a particular organ or tissue in the disposition of endogenous or exogenous chemicals (Mehendale, 1984).

ECGs were recorded from isolated toad's hearts that were directly perfused with 5µg/ml of *O. brevis* extract where it had pronounced effects on the myocardial activity. Negative chronotropic and dromotropic effects were noticed after *O. brevis* extract application, indicating a significant decrease in the heart rate (bradycardia) accompanied by an elongation of the P-R interval indicating increasing of conduction time. In the mean time, a positive inotropic effect represented as an increase in the R wave amplitude was recorded after 10 minutes of *O. brevis* application of the used dose, indicating an increase in the force of ventricular contraction.

In an attempt to reveal the mechanism of action of *O. brevis* extract, application of verapamil, a selective antagonist of L-type Ca^{2+} channels, has completely abolished the positive inotropic activity induced by *O. brevis* extract indicating a direct effect of *O. brevis* extract on the Ca^{+2} channels of myocardial fibers. Neither, atropine as a muscarinic cholinergic antagonist, nor propronolol could abolish the negative chrontropic and inotropic effects, which approved the above mentioned notice about the direct myogenic effect on the myocardium and exclude a neurogenic mechanism of action of the toxin (Nabil et al., 2004).

The positive inotropic effects as well as negative chronotropic and dromotropic actions of *O. brevis* extract on the heart might be result of binding to and inhibition of sarcolemmal Na^{+}-K^{+} ATPase. However, Inhibition of the Na^{+} pump causes an intracellular accumulation of Na^{+}, thus reducing Ca^{2+} removal from cytoplasm and/ or promoting reverse mode of Ca^{2+} influx via the Na^{+}/Ca^{2+} exchanger. This subsequently leads to an increase in Ca^{2+} accumulation in the sarcoplasmic reticulum (SR) inducing positive inotropic effect and causing SR Ca^{2+} overload in the case of toxicity(Lee, 1985).

Calcium ions are the most versatile ionic messengers. Where, stimulation of the outer surface of a muscle cell triggers thousands of Ca^{2+} releases from SR into the cytosol that increases intracellular calcium levels, creating a Ca^{2+} signal which activates calcium-sensitive proteins which in turn causes contraction of myofilaments via binding to the troponin component, this in turn results in a positive inotropic action (Silverthorn et al., 2010). Excessive calcium concentration in the cell may damage it or even cause it to undergo apoptosis or death by necrosis, associated with cardiac arrhythmias (Boron et al., 2003).

It was evidence that sinus abnormalities were the most frequent disorders followed by different degrees of Atrioventricular blocks reported after direct perfusion of isolated toad‘s heart with 5µg/ml *O. brevis* extract. This indicates that the toxic myogenic effects on the myocardium are mostly directed to both Sinoatrial (SA) and atrioventricular (AV) nodes. Furthermore, striking effects on ECG were noticed taken from perfused isolated toad‘s heart as a group of cardiac disorders. These pathologic cases included S-T segment depression, sinus arrhythmia, severe bradycardia, heart block and ectopic beats, which are often a feature of myocardial infarction (Julian et al., 2000 and Hussein, 2003). These observations confirm the direct mechanism of action of *O. brevis* toxin (Mohammed et al., 2011).

The mechanism of toxicity of many compounds requires formation of ROS, including superoxide anion, hydrogen peroxide, superoxide radicals and hydroxyl radical. Reactive oxygen species (ROS) are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function, or excessive quantities, the state called oxidative stress (Silverthorn et al., 2010). They are capable to react with proteins, nucleic acids, lipids and or other molecules leading to changes in their structures and consequently damage of cells. Fortunately, the cells are able to protect themselves against oxidative stress, as they develop numerous defensive mechanisms based on the antioxidative enzymes activity and action of low molecular antioxidants such as glutathione (Mates, 2000).

As lipid peroxidation (LPO) and oxidative stress play a key role in the pathogenesis of many diseases, the effect of *O. brevis* extract as free radical inducer was evaluated through their effect on lipid peroxidation (TBA reactants), endogenous antioxidants and morphological of erythrocyte, through in vitro study.

Marked changes were observed on lipid peroxidation, activity of antioxidants (GSH, CAT and SOD), in blood after in vitro incubation with 1µg/ml *O. brevis* extract. However, a significant increase in thiobarbituric acid reactive substances (TBARS) concentration after incubation with *O. brevis* extract was noticed. This indicates that membrane lipids were attacked by reactive oxygen species to form a carbon radical that reacts with free radical and results in a peroxyl radical, thus generating lipid peroxides (Domenico et al., 2000). In the mean time, imbalances in antioxidant defense as the consequence of increase of free radical generation stimulated by *O. brevis* extract were reported by (Fayun, et al., 2007 & Pasupathi et al., 2009 and Mohammed et al., 2010). This observation is in agreement with the changes caused by microcystin-LR (MC-LR) and anatoxin-a on LPO level and antioxidants (Mitrovic et al., 2004).

Glutathione (GSH) is the most abundant intracellular thiol-based antioxidant, prevalent in millimolar concentrations in all living aerobic cells. Its function is mainly as a sulfhydryl buffer, but GSH also serves to

detoxify compounds either via conjugation reactions catalyzed by glutathione S-transferases (Armstrong, 1997) or directly, as is the case with hydrogen peroxide in the glutathione peroxidase (GPx) catalyzed reaction (Van Bladeren, 2000). GPx may catalyze the reduction of H_2O_2 using glutathione as a substrate (Mates et al., 1999).

In the current study, imbalanced changes in GSH level were noticed. However, a decrease in GSH level followed by an increase is correlated with different time and dose treatment. GSH depletion could be related to its involvement in the detoxification of the deleterious effects of the increase free radicals produced within the cell (Moussa, 2009 and Nabil et al., 2006). Then, Increases in the level of GSH can play a critical role in the protection of cells from oxidative stress. However, the capacity to increase GSH synthesis in response to increased demands on GSH utilization is thought to be an important determinant of cell survival (Maher, 2005). This observation coincides with Halliwell and Gutteridge (1993) who observed that although severe oxidative stress, which is defined as an imbalance between the production and removal of reactive oxygen species, can cause a decrease in GSH were a number of reports have shown that moderate stress often increases GSH. This increase is likely to provide protection of cells from both the ongoing stress and from subsequent, more severe stress.

Many compounds initially decrease GSH levels before eventually leading to an increase well above control levels (Maher, 2005). However, treatment with compounds that decrease GSH can lead to a transient increase in GSH levels. This observation coincides with Maher (2005) who noticed that the increase in GSH levels correlates in a time and dose dependent fashion with an increase in the level of Glutamate cysteine ligase **(**GCL) which catalyzes the first and rate-limiting step in GSH biosynthesis (Dickinson et al., 2004)

It is well know that, catalase and superoxide dismutase are able to scavenge reactive oxygen species (ROS) such as the superoxide anion and strong oxidant hydrogen peroxide (Sicin´ska et al., 2006& Reiter et al., 2000**).** Catalases of many organisms are mainly heme-containing enzymes (Quick et al., 2008). The predominant subcellular localization in mammalian cells is in peroxisomes, where catalases catalyze the dismutation of hydrogen peroxide to water and molecular oxygen (Halliwell, 1999)

Moreover, a significant increase in catalase activity after incubation of 1µg/ml O.brevis extract for 4 and 6 hours was noticed. This observation is in agreement with Qiu et al., (2009) who proved that microcystin-LR induced oxidative stress by increasing lipid peroxidation and activity antioxidants (CAT and SOD) after I.V administration in rats. Sicin´ska et al., (2006) noticed that no change was observed on catalase activity after incubated human blood with low doses of microcystin-LR on human blood for 1, 6, 12, 24 hours.

On the other hand, the present *in vitro* study revealed that erythrocyte SOD activity highly declined after treatment with O. brevis extract compared with control group coinciding with that noticed in the *in vivo* experiments (Mohammed et al., 2011). SOD converts superoxide anion radicals produced in the body to hydrogen peroxide. SOD activity may be decreased either due to direct damage of protein structure by *O. brevis* extract or could be consumed by increasing amount of hydrogen peroxide (Sicin´ska et al., 2006). Disturbance in removal of superoxide radical provokes further damage of the SOD enzyme and causes the increase of the level of H_2O_2 (O¨ ztu¨rk and Gu¨mu¨s¸lu¨, 2004). The above mentioned results confirm the previous work of Sicin´ska et al., (2006) who noticed that Microcystin-LR decreased the activity of SOD and increased lipid peroxidation in blood samples. On the contrary, Mitrovic et al., (2004) showed that anatoxin-a increased catalase activity and caused formation of reactive oxygen species, increased SOD activity and slightly increased lipid peroxidation. Accordingly, it can be concluded here that *O. brevis* toxin extract induced a disturbance in antioxidants system and increased lipid peroxidation consisting in oxidative stress.

The oxidative stress resulted from ROS generation can lead to a decrease in calcium responsiveness of myofilaments either directly by oxidative modification of contractile protein, or indirectly, by causing Ca^{2+} overload. There are at least two mechanisms whereby ROS can produce calcium overload: i) ROS can promote sarcolemmal damage, with consequent loss of selective permeability, impairment of calcium- stimulated ATPase activity and calcium transport out of cell, and ii) ROS can impair calcium stimulated ATPase activity and calcium transport in the sarcoplasmic reticulum, resulting in decreased calcium sequestration and increased free cytosolic calcium (Bolli and Marban, 1999)**.**

In addition, enhanced lipid peroxidation may affect the lipid moiety of one or more of the subcellular organelles that contribute to the process of contraction other than those affecting Ca^{2+} homeostasis and increased free cytosolic calcium (Jamall,1987). These suggestions may offer an explanation of cardiotoxicity induced by *O. brevis* extract in isolated toad's heart with significant negative chronotropic and dromotropic effect on atrioventricular conduction system and stimulatory inotropic effect of ventricular contraction. An *in vivo* study might be needed to confirm these suggestions.

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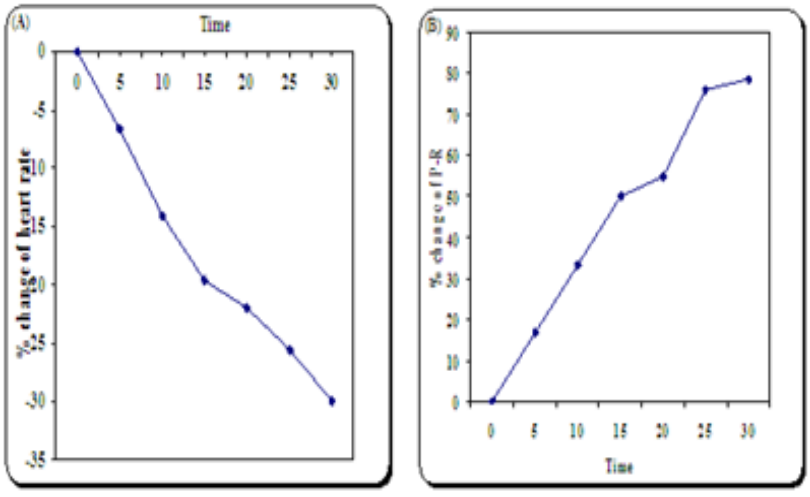
Table 1 Effect of direct perfusion with (5µg/ml) *O. brevis* extract on the heart rate (HR), conduction time (P–R interval), amplitude of ventricular contraction (R-amplitude), and repolarization voltage (T-amplitude) of isolated toad hearts

Time (min)	Heart rate (beat/min)	P-R interval (msec)	R- Amplitude (mV)	T- Amplitude (mV)
0	53.1 ± 1.6	420 ± 13.3	1.94 ± 0.09	0.82 ± 0.19
5min	49.6 ± 1.4¥	490 ± 17.9¥	2.5 ± 0.18§	0.92 ± 0.11
10min	45.6 ± 1.7¥	560 ± 16.3¥	2.84 ± 0.29§	1.38 ± 0.13§
15min	42.7 ± 0.8¥	630 ± 21.3¥	2.82 ± 0.22§	1.64 ± 0.17§
20min	41.4 ± 0.9¥	650 ± 26.8¥	2.66± 0.23§	1.77 ± 0.19§
25min	39.5 ± 1.3¥	740 ± 30.6¥	2.44 ± 0.22*	1.6 ± 0.18§
30min	37.2 ± 1.2¥	750 ± 37.3¥	2.3 ± 0.24	1.56 ± 0.2*

Values expressed as mean ±SE (n =10 /group).
Significantly different between self control (0- time) and treated groups (5, 10, 15, 20, 25 and 30 min) using Student's paired t-test. *(P≤ 0.05), § (P≤ 0.01), ¥ (P≤ 0.001).



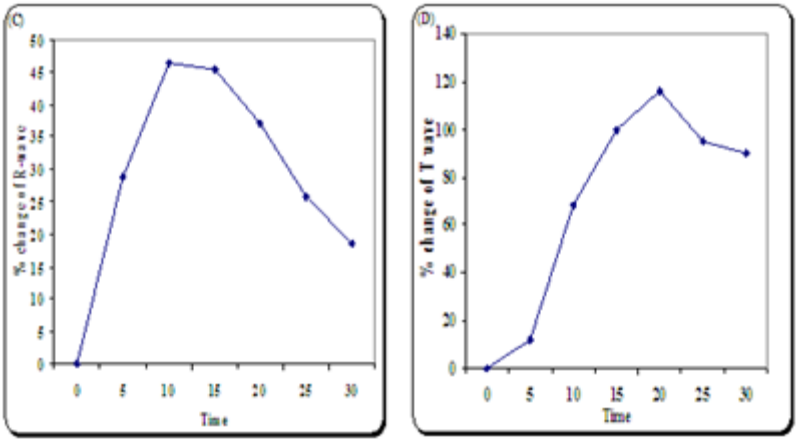


Figure1. Percentage of change of (A) heart rate and (B) P-R interval, (C) R amplitude, (D) T- amplitude of isolated toad's heart after perfusion with 5µg/ml *O. brevis* extract. (n=10/ group).

Table 2: Percentage of incidence of ECGs abnormalities recorded from isolated toad's heart after direct perfusion with 5µg/ml of *O. brevis* extract (n=10/group).

ECG abnormalities	Percentage of incidence (%)
Sinus abnormalities	
Atrioventricular block	33%
Sinus arrhythmia	40%
Systole	31%
Ectopic beats	
Junctional escape	20%
Ectopic beat	53%
P wave abnormalities	
Inverted P-wave	3%
Peaked P-wave	7%
QRS wave abnormalities	
Wide QRS complex	33%
Negative QRS deflection	10%
ST segment abnormalities	
S-T segment depression	44%

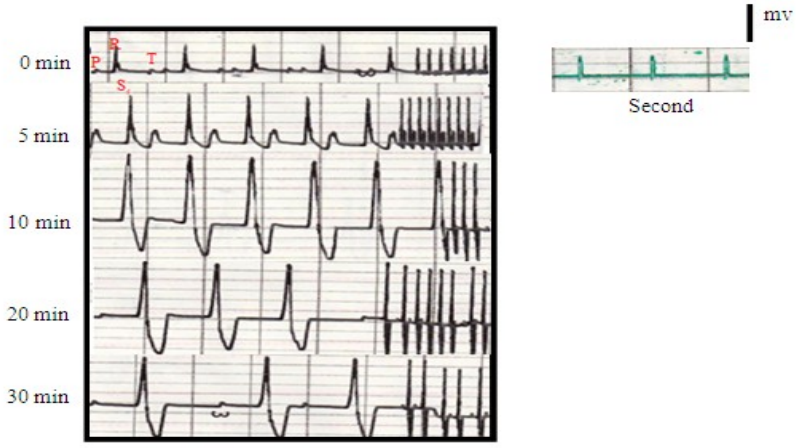


Plate 1: ECG traces showing the effect of direct perfusion of isolated toad's heart with 5µg/ml of *O. brevis* extract on the electrocardiogram of isolated toad's heart at different time intervals.

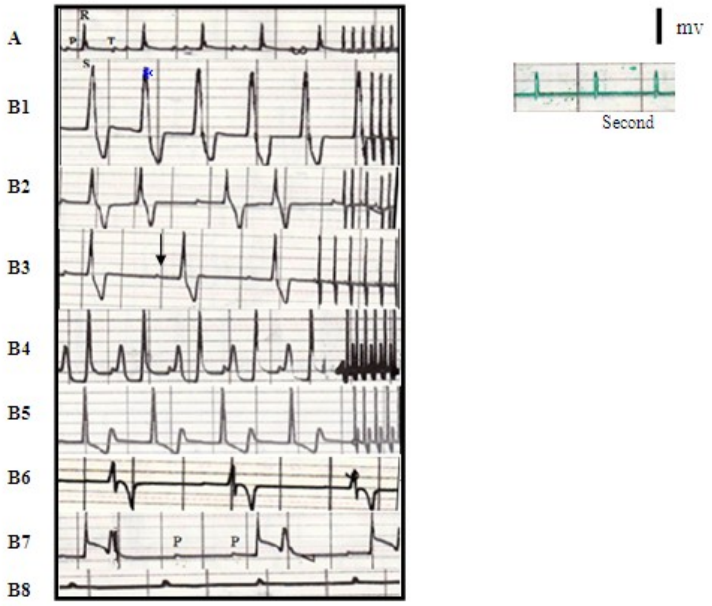


Plate 2: ECGs recorders showing examples of cardiac disorders induced by direct perfusion of isolated toad's heart with 5µg/ml of *O. brevis* extract.

A- Before treatment. B- After treatment.
1- Wide QRS-Wave. 2- Sinus arrhythmia and *ST- segment depression.
3- First degree block 4- Peaked P- wave
5- Ectopic beats. 6- Negative QRS deflection
7- Second degree block 8- Complete heart block.

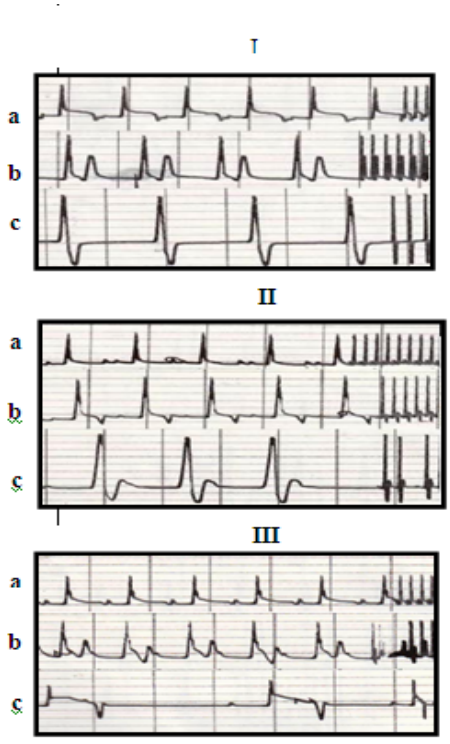


Plate 3: ECGs recordings showed the mechanism of 5µg/ml *O. brevis* extract action on isolated hearts.
I-4µg/ml of Atropine sulfate, II-5µg/ml of propranolol and III- 5µg /ml of verapamil
a) Before treatment. b) 10-min after treatment.
c) After adding blocker.

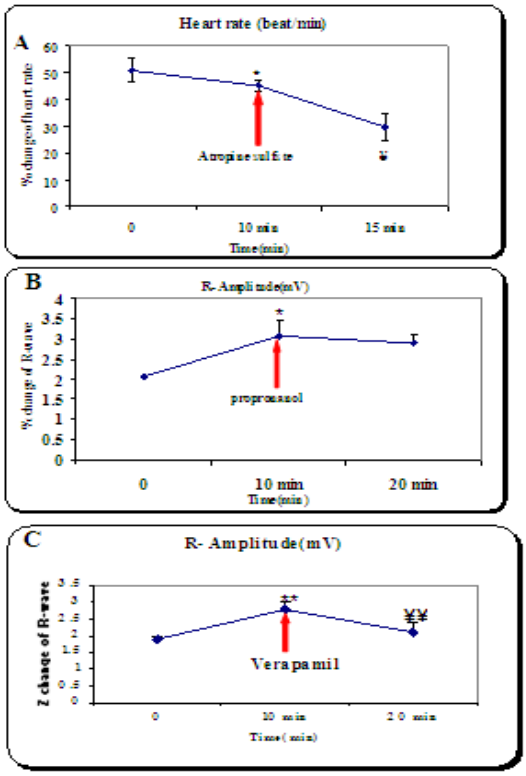


Figure2. Mechanism of action of 5µg/ml *O. brevis* extract on isolated heart. (A) 4µg/ml of Atropine sulfate, (B) 5µg/ml of propranolol and (C) 5µg/ml of verapamil Values represent mean ± SD (n = 5/group). *Significantly different from zero value by student's paired t-test ($P < 0.001$). ¥ Statistically different from the value before blockers application student's paired t-test

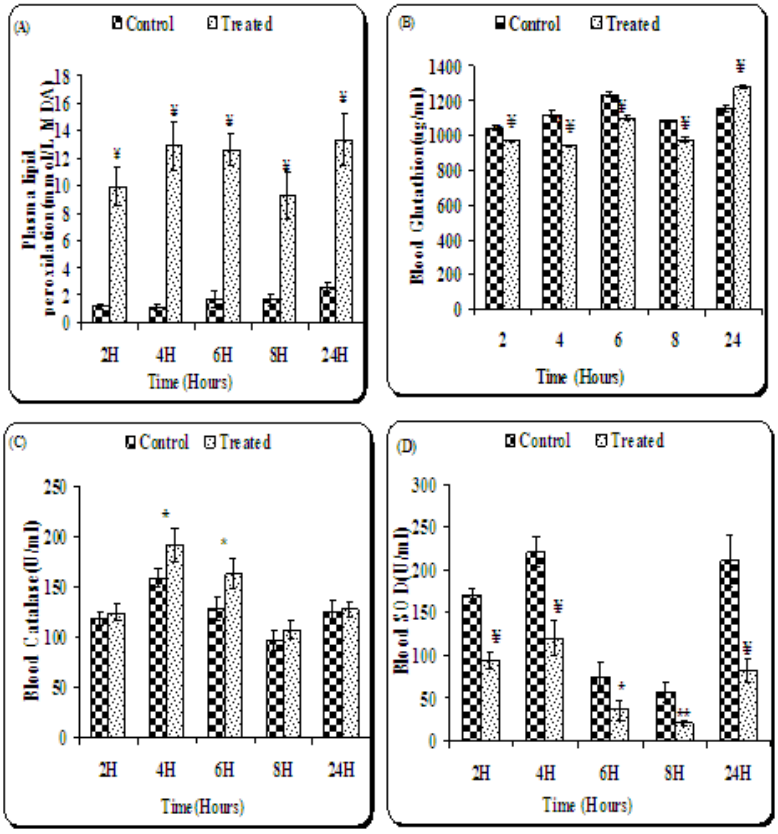


Figure 3. Effect of direct incubation of blood with 1µg/ml *O. brevis* extract on (A) Plasma lipid peroxidation, (B) Blood glutathione. (C) Catalase and (D) Superoxide dismutase respectively. Values represent means ± S.E (n=10/ group).* (P≤ 0.05) ** (P≤ 0.01) ¥ (P≤ 0.001), significantly different from control group, student's unpaired t-test.