

Cymbopogon citratus 80% Methanolic Leaf Extract Inhibit Acetyl-Cholinesterase on Mice Model

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Abstract

Oxygen is a unique element indispensable for life. Free radicals are formed as a consequence of ATP (adenosine triphosphate) production by the mitochondria in oxidative phosphorylation due to use of oxygen by cells to generate energy. Most of the by-products formed as a result of this biochemical change are reactive oxygen species (ROS) as well as reactive nitrogen species (RNS). Delicate balance between the two antagonistic effects is among the vital aspects of life. ROS and RNS exert beneficial effects on cellular responses and immune function at low or moderate concentrations. Therefore, the aim of this study is to evaluate the cholinesterase inhibitory effect of *Cymbopogon citratus* 80% (leaf) extract. The leaf was extracted with 80% methanol. A toxicity study was carried out on mice. Cholinesterase inhibitory activities were also evaluated on the same mice using Ellman's method. Results revealed high cholinesterase inhibitory activities of the crude extract with high significant differences at $P < 0.001$) between the group that were treated with crude extract only, group treated with crude extract and exposed to arsenic and group that were exposed to arsenic only as well as group that are maintained in complete media. It can be concluded that low toxicity and high cholinesterase inhibitory effect of the crude extract is responsible for its therapeutic effects of this crude extract. Toxicity screening of this crude extract on a mammal such as rat to reaffirm their toxicity profile is recommended. Antioxidant screening as well as isolation of bioactive compounds present in this plant part is strongly recommended.

Keywords: Cholinesterase, *Cymbopogon citratus*, Ellman's method, mice toxicity.

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INTRODUCTION

Arsenic is an environmental contaminant and its presence in water can lead to environmental pollution [1]. Long time exposure to arsenic can predispose to immense health distress as it accounts for the increased risk of various disorders such as cardiovascular abnormalities, diabetes mellitus, neurotoxicity, and nephrotoxicity [2]. Similarly, arsenic intoxication is reported to be among the serious causes of hepatic dysfunction and hepato-cellular toxicity [3]. In addition, carcinogen induction particularly skin, bladder, lung, hepatic and brain tumor were shown to be caused as a result of long time exposure to arsenic [4]. Several researches also documented the potential of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present in the plasma membrane of vascular endothelial cells and vascular smooth muscle cells (VSMC) to be stimulated by arsenic [5]. Over stimulation of NADPH oxidase causes increased reactive oxygen species (ROS) such as superoxides and hydrogen peroxide generation leading to cytotoxicity

effect [6]. To find solutions to these problems, medicinal plant *Cymbopogon citratus* leaf was obtained, extracted and evaluated for its effects on preventing diseases caused by accumulation of free radicals.

Cymbopogon citratus, Stap (Lemon grass) is a widely used herb in tropical countries, especially in Southeast Africa [7]. The essential oil of the plant is used in aromatherapy. The compounds identified in *Cymbopogon citratus* are mainly terpenes, alcohols, ketones, aldehyde and esters. Some of the reported phytoconstituents are essential oils that contain Citral α , Citral β , Nerol Geraniol, Citronellal, Terpinolene, Geranyl acetate, Myrcene and Terpinol Methylheptenone [8]. The plant also contains reported phytoconstituents such as flavonoids and phenolic compounds, which consist of luteolin, isorientin 2'-O-rhamnoside, quercetin, kaempferol and apigenin [9]. Studies indicate that *Cymbopogon citratus* possesses various pharmacological activities such as anti-amoebic, antibacterial, antidiarrheal, antifilarial, antifungal and anti-inflammatory properties [10].

Various other effects like antimalarial, antimutagenicity, antimycobacterial, antioxidants, hypoglycemic and neurobehavioral have also been studied. These results are very encouraging and indicate that this herb should be studied more extensively to confirm these results and reveal other potential therapeutic effects. Therefore, the aim of this research is to evaluate the free radical protective effects of *Cymbopogon citmiceus* 80% leaf methanol extract by studying its effect on acetyl-cholinesterase enzyme on mice brain. To achieve these mice which is a mammalian vertebral fish and physiologically closer to human being is used in this research.

Mice have been used as model organisms to study human biology due to the genetic and physiological similarities between the two species [11]. Moreover, mice and humans have evolved in and become adapted to different environments and so, despite their phylogenetic relatedness, they have become very different organisms [12]. Mice often respond to experimental interventions in ways that differ strikingly from humans [13].

MATERIALS AND METHODS

Ethical Approval

This study was scrutinized and approved by the University Committee on Medical and Scientific Research Ethics, in accordance with National Health Research Ethics Committee of Nigeria (NHREC) directive.

Plants collection and identification

The plant leaf was obtained from Department of Veterinary Pharmacology and Toxicology garden at city campus Usmanu Danfodiyo University Sokoto (UDUS). Botanists from Biological department UDUS authenticate the plant leaf and allocate voucher number.

Plant Extraction

The stem cleaned and cut into small pieces with anvil pruner, (UK). The leaf was allowed to air dried for two weeks at room temperature (26 ± 1 °C) in lab and crushed to smaller sizes. Two hundred (200) g of sample (leaf) was soaked for 3 days in 1000 ml 80% methanol in flat bottom flasks (Sigma Aldrich, USA). Crude mixture was shaken every day at 26 °C to ensure adequate dilution and extraction. The process was repeated three times to extract most of the bioactive compound present in the leaf. The extract was then filtered with Whatman filter paper (1.5 Sigma Aldrich, USA) and then concentration to semi-solid form at 42 °C with a rotary evaporator (IKA® RV 10, USA). The resultant crude extract obtained was then weighed and transferred into sample bottles and stored at 4 °C until required.

Percentage yield was calculated as the weight of the filtrate divided by the total weight of the

ground powder in percentage. Yield (%) = $[\text{wt of extract (g)}/\text{wt of plant material (g)}] \times 100$.

Plants sample dilution and dose preparation

Stock solution was prepared by dissolving 100 mg of the crude extract into 1 ml of 100% DMSO (100 mg/L). DMSO was used to solubilize the crude extract in aqua solvent. Preparation of sub-stocks in mg/ml was carried out by diluting the stock solution with distilled water to the concentration of interest using two-fold serial dilution (mg/ml) in sample bottle (Sigma Aldrich, USA). DMSO (vehicle) was maintained at 0.1% in all concentration of extract.

Toxicity Study

Healthy adult male and female mice, aged 1-2 month, weighing 80-100g, were purchased from animal's house at Faculty of pharmaceutical science Usmanu Danfodiyo University Sokoto as reported by the OECD guideline for testing of chemicals (OECD, 2013). The animals were acclimatized for 2 weeks at Biochemistry lab Faculty of Veterinary Medicine (26 ± 2 °C; 12: 12 hour dark/light cycle), fed with commercial feed ad libitum.

Acute toxicity test of arsenic on mice

Group-I –Five mice were exposed to arsenic for 60 minute for 2 days using nebulizer.

Group-II –Five mice were exposed to arsenic for 40 minute for 2 days using nebulizer.

Group-III –Five mice were exposed to arsenic for 20 minute for 2 days using nebulizer.

Group-IV –Five mice were exposed to arsenic for 1 minute for 2 days using nebulizer.

Group-V –Five normal control mice were not exposed to arsenic.

Chronic toxicity test of arsenic on mice

Group-I –Ten mice were exposed to arsenic for 40 minutes for 7 days using nebulizer

Group-II –Ten mice were exposed to arsenic for 30 minutes for 7 days using nebulizer

Group-III –Ten mice were exposed to arsenic for 20 minutes for 7 days using nebulizer

Group-IV –Ten mice were exposed to arsenic for 10 minutes for 7 days using nebulizer

Group-V –Ten normal control mice were not exposed to arsenic.

Acute toxicity test of crude extract on mice

Group-I –Five mice were given 2000 mg/kg of crude extract orally for 2 days

Group-II –Five mice were given 1000 mg/kg of crude leaf extract orally for 2 days

Group-III –Five mice were given 500 mg/kg of crude leaf extract orally for 2 days

Group-IV –Five normal control mice were given commercial feed and distilled water for 2 days

Chronic toxicity test of crude extract on mice

Group-I –Five mice were given 1000 mg/kg of crude extract orally for 14 days
 Group-II –Five mice were given 500 mg/kg of crude extract orally for 14 days
 Group-III –Five mice were given 250 mg/kg of crude extract orally for 14 days
 Group-IV –Five mice were given 125 mg/kg of crude extract orally for 14 days
 Group-V –Five mice were given 62.5 mg/kg of crude extract orally for 14 days
 Group-VI –Five normal control mice were given commercial feed and distilled water for 2 days

At the end of the experiment, animals were sacrificed with aid of chloroform in aqueous solution. Each mice was placed on dorsal recumbence incision was made using scalpel blade and the skull was dissected and brain was gently removed and washed with distilled water.

Total Protein Estimation

The protein content was determined in homogenize mice brain using Bradford method in which bovine serum albumin (BSA) (7.81-1000 µg/mL) was used as standard. Tris-HCl buffer prepared with 1% Triton X and 0.1 PMSF pH 7.4, BSA, Broad ford reagent, 96-well micro-titer plates, Micro-pipetter and tips, Dissecting needle, Plate reader with 590 or 595nm filter Berthold Technologies GmbH & Co. KG. Standard curve, using BSA was plotted. 1 ml stock solutions of 1000 µg BSA/200µl Tris-HCl (10 mg/200 ml) and freeze, until needed. Thaw and dilute with Tris-HCl and diluted at different concentration from 0 to 1000 µg/mL Add 200 ul of sterile phosphate saline (PBS) minus the volume of extract to each well.

After running the assay, the standard curve was used to determine the concentration according to OD values.

Determination of cholinesterase activity of plant extract

Screening of cholinesterase activities of the crude extract on mice brain was carried out. Animals were sacrificed with aid of chloroform in aqueous solution. Each mice was placed on dorsal recumbence incision was made using scalpel blade and the skull was dissected and brain was gently removed and washed with 50 mM Tris-HCl buffer, weighed and homogenized in a scope bottle with the aid of (Polytron PT-6100, tissue homogenizer USA). Tris-HCl buffer was prepared with 1% Triton X and 0.1% phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich

and used as homogenizing solvent. Sample was centrifuged at 12,000 x g for 20 minutes with (GRACE High Speed Refrigerated Centrifuge India). The supernatant was transfer into separate tube and used as enzyme source.

Based on Ellman's method, anti-cholinesterase (ChE) activity was measured using a modified 96-well microplate assay. The hydrolysis of the substrate acetylthiocholine by enzyme reaction results in the production of thiocholine. Thiocholine reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine which was measured at 405 nm. 50 mM Tris-HCl pH 7.4 was used as a buffer throughout the experiment. Acetylcholinesterase (Ache) used in the assay was from the homogenized exposed mice brain. The 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), Acetylcholine (ATC), Butyrylcholine (BTC), and Propionylcholine (PTC) were dissolved in 50mM Tris-HCl. In the 96-well plates, 210 µL of Tris-HCl buffer (pH7.4), 20 µL of 0.1 mM DTNB, and 10 µL of AChE (54 mg/ml) were transferred and incubated for 15 min at 28°C. 10 µL of ATC, BTC or PTC (2.5 mM) were then added to the mixture and incubated for 10 minutes. Reading was taken at 405 nm using a micro plate reader (Tecan multimode microplate United Kingdom) at fluorescent excitation of 485 nm and 535 nm emission.

RESULT

Crude extract yield of

The result of the percentage yield of *Cymbopogon citratus* leaf after extraction, evaporation and concentration with a rotary evaporator at 42 °C was 13.42%.

Acute toxicity study of arsenic

The result of sub-acute toxicity test of arsenic on mice shows high mortality in groups exposed arsenic for 10 minutes. Up to 80% of the mice survived at 24 hours post exposure and only 13.3% survived at 48 hours post exposure in groups that were exposed for 20 minutes of arsenic. Survival analysis of groups that were exposed for 40 minutes shows only 20% of the mice survived at day 1 post exposure and all mice die at 48 hour post exposure. All mice exposed for 60 minutes of arsenic die at 24 hour post exposure. Comparison of Survival Curves using Log-rank (Mantel-Cox) Test and Gehan-Breslow-Wilcoxon Test at degree of confident (df) 1 shows high significant difference with $p < 0.001$ between the groups that were maintained in 0.1% DMSO and the groups that were exposed to different concentration of the arsenic (Figure-1).

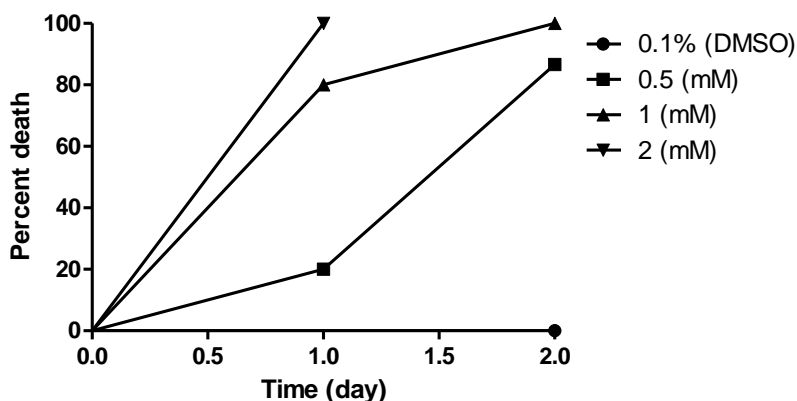


Fig-1: Sub-acute toxicity effect of arsenic on the survival rate of adult mice exposed to arsenic 10-60 minutes for 2 days. The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean \pm SEM from three independent experiments. ***P<0.001 represented significantly different values of the percentage survival

Chronic toxicity study of arsenic

The result of sub-chronic toxicity test of arsenic on Mice shows high mortality with high concentration of the arsenic. Up to 66.6% of the mice survived at day 6 of the experiment and all die at day 7 post exposures in groups that were exposed for 10 minutes. In groups exposed for 20 minutes, 75% of the mice survived at day 4, 25% survived at day 5 of the experiment and all die at day 6 post exposure. Survival analysis of groups that were exposed to 0.75 mM shows 85.7 % survived at day 2, 71.4% survived at day 3,

14.3% survived at day 4 of the experiment and all mice die at die at day 5 post exposure. All mice exposed to 1.75 mM arsenic die before 24 hour post exposure. Comparison of Survival Curves using Log-rank (Mantel-Cox) Test at of df 1 and Gehan-Breslow-Wilcoxon Test at of df 4 shows high significant difference with $p < 0.001$ between the groups that were maintained in 0.1% DMSO and the groups that were exposed to different concentration of the arsenic (Figure-2).

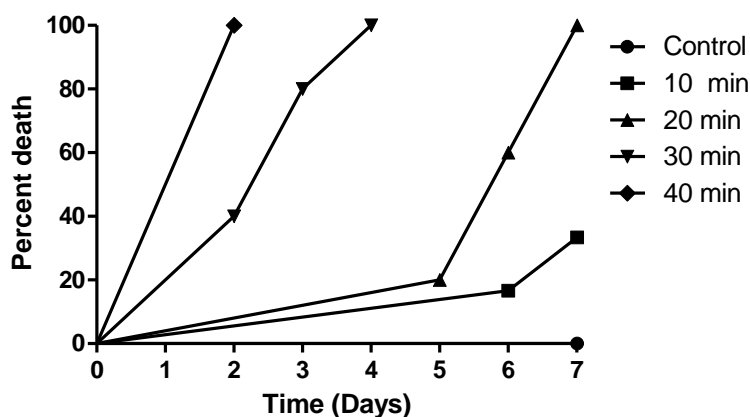


Fig-2: Sub-acute toxicity effect of arsenic on the survival rate of adult mice exposed to arsenic 10-40 minutes for 14 days. The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean \pm SEM from three independent experiments. ***P<0.001 represented significantly different values of the percentage survival

Acute toxicity study of the crude extract

The result of acute toxicity test of crude on mice shows that all mice that were not exposed to the crude extract survived at day 2 of the experiment. Up to 100% of the mice survived at 84 hours post exposure in group exposed to 500 mg/kg. About 1000% of the mice survived the first 24 hours and only 20% survived at 48 hours post exposure in groups that were exposed to 1000 mg/kg of the crude extract. Survival analysis also shows that all the mice exposed to 2000 mg/kg die at 24

hours of the experiment. Comparison of Survival Curves using Log-rank (Mantel-Cox) Test at df 5 shows slight significant different at $p < 0.01$ between the group that were maintained in 0.1 DMSO and those that were exposed to different concentration of the crude extract. Gehan-Breslow-Wilcoxon Test to compare the survival curves at df 1 shows high significant difference with $p < 0.0001$ between the groups that were maintained in 0.1% DMSO and the groups that were exposed to different concentration of the extract (Figure-3).

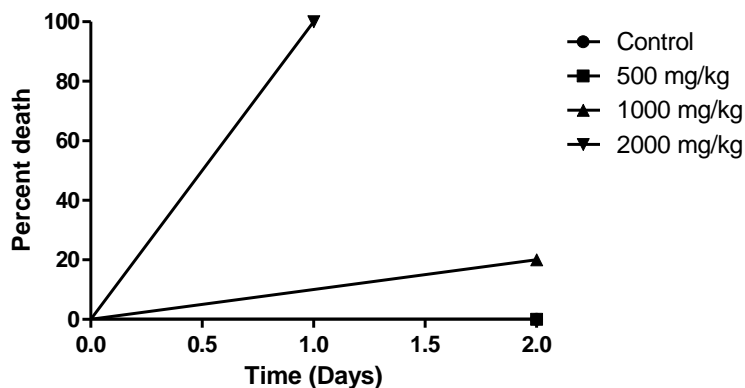


Fig-3: Acute toxicity effect of crude extract on the survival rate of adult Mice treated with different concentration (500-2000 mg/kg). The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean ± SEM from three independent experiments. ***P<0.001 represented significantly different values of the percentage survival

Chronic toxicity study of crude extract

The result of chronic toxicity test of crude extract on mice shows up to 100% of the mice exposed to 62.5 mg/kg concentration survived the last 14 day of the experiment. In group exposed to 125 mg/kg, 80% survived at day 12, 60% survived at day 14 of the experiment. In groups that were exposed to 250 mg/L crude extract concentration, 80% survived at day 10, 40% survived at day 11, 20% survived at day 12, and the remaining die at day 13 of the experiment respectively. The result also shows up to 80% survived at day 7, 60% survived at day 8 and the remaining mice

die at day 10 of the experiment on the groups exposed to 500 mg/kg of the crude extract. Survival analysis of the groups exposed to 1000 mg/kg crude extract also revealed 80% of the mice survived at day 4, 40% survived at day 5 all remaining mice die at day 7 of the experiment. Comparison of Survival Curves using Log-rank (Mantel-Cox) Test at df 7 and Gehan-Breslow-Wilcoxon Test to compare the survival curves at df 1 shows high significant difference with p< 0.0001 between the groups that were maintained in 0.1% DMSO and the groups that were exposed to different concentration of the arsenic (Figure-4).

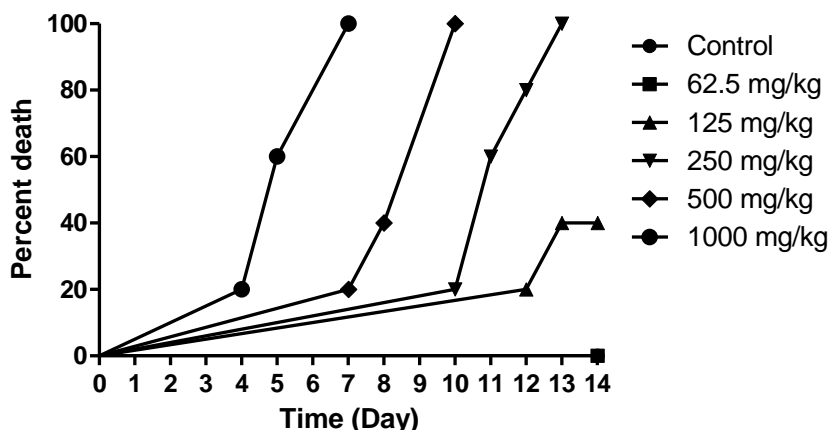


Fig-3: Chronic toxicity effect of crude extract on the survival rate of adult Mice treated with different concentration (100-400 mg/L). The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean ± SEM from three independent experiments. ***P<0.001 represented significantly different values of the percentage survival

Total Protein Content

Result of total protein content shows high protein content in the control groups followed by the groups that were treated with 62.5 mg/kg crude extract only then groups that were treated with 62.5 mg/kg crude extract and exposed to arsenic for 20 minutes compared to the groups that were exposed to arsenic 20

minutes only. There was significant difference at p<0.001 between the control groups and the groups that were treated with crude extract only compare to the groups that were exposed to arsenic only. There is no significant different between mice that were exposed to arsenic only and those that were treated with the crude extract and then exposed to arsenic (Figure-5).

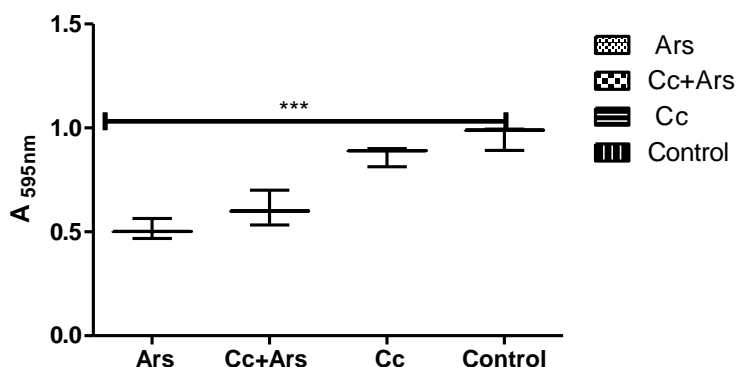


Fig-5: Effects of *Cymbopogon citratus* (leaf) 35 µg/mL extract and arsenic (0.15 M) on total protein content extracted from the brain of adult Mice. ***P<0.001 represented significantly different values from an arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = arsenic and Cc = *Cymbopogon citratus* (leaf) extract

Acetyl-cholinesterase inhibitory assay

Result of acetyl-cholinesterase inhibition shows high activities in control groups. Increased activities was also observed in groups that were treated with crude extract only as well as groups that were treated with crude extract and exposed to arsenic

compared to the groups that were exposed to arsenic only. There was significant difference at $p < 0.001$ between the mice that were treated with arsenic compare to the control group, those that were treated with the extract only as well as those that were treated with the extract and exposed to arsenic (Figure-6).

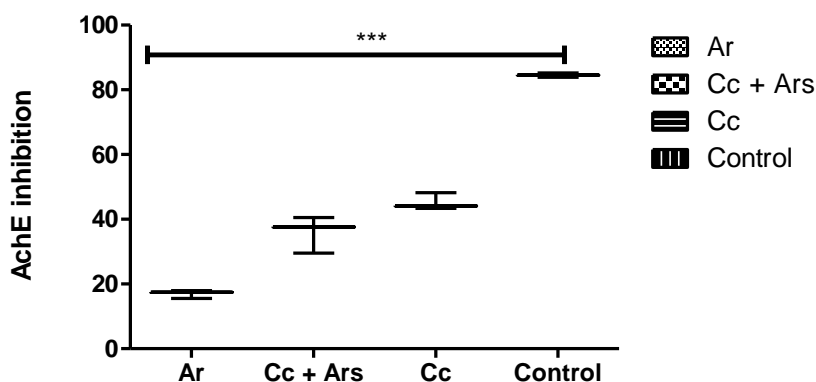


Fig-6: Acetyl cholinesterase inhibitory effects of *Cymbopogon citratus* leaf extracts against Arsenic was measured using DTNB, Acetylcholine iodide substrate with a microplate reader at 405 nm. ***P<0.001 represented significantly different values from an Arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = arsenic and Cc = *Cymbopogon citratus* (leaf) extract.

DISCUSSION

Commonly used lab solvents for extraction of bioactive plant component are methanol, ethanol, acetone and ethyl acetate [14]. Previous studies reported high of phenolic compounds with acetone compared to remaining solvents in an experimental extraction of fruit and vegetable [15]. However, high phenolic yield with methanol in leaf extracts compare to acetone, hot water and chloroform leaf extracts has also been documented [16]. Variation in polarity may be among the major factors that affect the increase affinity and extraction of bioactive compound from plant [17]. Hence, considering the high ability of polar solvent to extracting large quantities of bioactive compounds and its adopted used by herbalist, 80% methanol was choosing as extraction solving. The result of percentage yield obtained after extraction of 200 g was 13.42%. This result is not in agreement with the finding reported by Lay *et al.*, 2014 which shows high

yield of 331 g (6.55% w/w) following extraction of *Lophira lanceolata* leaf with 80% methanol.

The result of sub-acute toxicity study of arsenic on mice shows that only groups that were not exposed to arsenic (control) and the groups that were treated with arsenic low dose (10 minute exposure) survived. Deaths were recorded in groups that were exposed for 40, 60 minutes of arsenic before the last day of the experiment. There is a significant difference ($p < 0.001$) among the groups that were exposed arsenic at different time and the control group. Chronic toxicity study also revealed above 50% survival rate in groups exposed for 10 and 20 minutes compare to the other groups. There is significant difference ($p < 0.001$) among the groups that were exposed to arsenic at different time and the control groups. There was no sufficient literature on the experimental effect of arsenic on mice, but several scientists reported it toxic effects in human

and rat. Arsenic has been discovered as one of human carcinogen especially in area where metal pollutant naturally occur in groundwater and unnaturally in mine waste sites and agricultural runoff [18]. Many studies have also reported arsenic's effect on embryological development and altered signaling pathways [19]. Studies have suggested that arsenic causes teratogenic defects, and it has been shown to cross the mammalian placenta, affecting developing embryos whose mothers undergo exposure [20].

Result of sub chronic toxicity effect show that, all mice exposed to 250 mg/kg die before day 12 of the experiment. Mice exposed to 62.5 and 125 mg/kg survived with little mortality. The calculated LC_{50} is 41.29 ± 0.9 mg/kg and there is significant difference ($p < 0.001$) among the groups that were exposed to different concentration of crude extract and the control group. There was no literature available on the toxicity effect of *Cymbopogon citratus* on mice, but Altaf *et al.*, 2013 reported no toxicity effect on acute experimental study with the same plant [21]. There were no observed histological changes in liver, no periportal necrosis of the hepatocytes, inflammation of lymphocytes and macrophages in both control and treated groups. No difference was observed in glomeruli or any other segment of kidney tubules when compared with their respective normal rats [22]. Toxicity effects of crude extracts are associated with the nature and concentration of secondary metabolites present [23].

Effects of crude extract 50 mg/mL and arsenic (0.15 M) on total brain protein content, acetylcholinesterase inhibition, butyryl-cholinesterase inhibition and propionyl-cholinesterase inhibition on mice was measured with fluorescent micro-plate reader. There are high significant differences between groups that were exposed to arsenic, groups that were treated with crude extract and the groups that were treated with crude extract for 24 hours and exposed to arsenic with $p < 0.001$. There was no published article on inhibitory effect of plant extracts on acetyl cholinesterase in mice. Cholinesterase or choline esterase is known to metabolize choline-based esters (acetyl choline) which function as neurotransmitter at neuromuscular junction to choline and acetic acid [24]. This allowed contraction and relaxation of muscle and impulse transmission across the nerves and invaded muscle. Imbalance of cholinesterase enzyme affects normal physiological activities of the nerve and muscles [25]. High concentration of this enzyme causes increase contraction of the muscle and overstimulation of nerves [26].

CONCLUSION AND RECOMMENDATION

It can be concluded that low toxicity and high cholinesterase inhibitory effect of the crude extract is responsible for the therapeutic effects of this crude extract. Toxicity screening of this crude extract on other

mammal such rat to reaffirm their toxicity profile are recommended. Antioxidant screening as well as isolation of bioactive compounds present in this plant part is strongly recommended.

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