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LEUCAS LAVUNDULIFOLIA AMELIORATES EXPERIMENTALLY INDUCED SODIUM ARSENATE TOXICITY IN INTESTINAL EPITHELIAL CELLS THROUGH ABROGATION OF OXIDATIVE STRESS

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ABSTRACT

Chronic exposure to arsenic leads to oxidative stress along with damage to several organs. The crude extract was designed to investigate the ameliorative properties of the *Leucas lavundulifolia* against arsenate induced cytotoxicity. Leaves are used as vegetable in part of the world. Intestinal cells were selected as a mode to analyze different aspects such as ROS, MMP, apoptosis, Chromatin condensation and DNA damage. DPPH and ABTS free radical scavenging assays were performed to determine the MTT and tryphan blue assays were done to evaluate the cytotoxicity. Inhibition concentration (IC_{50}) was found to be 48µM for arsenate. Best dose was found to be 125 µg/mL in intestinal epithelial cells. Administration of the extract reduced ROS, altered MMP, reduced chromatin condensation and DNA damage effectively ($p<0.05$). These can be attributed to phenolic compounds and flavonoids of the crude extract. Results obtained indicates the antioxidant properties and future applications of the *Leucas lavundulifolia* against arsenic induced toxicity.

KEY WORDS : DPPH, ABTS, ROS, MMP, *Leucas lavundulifolia*

INTRODUCTION

High accumulations of heavy metals like arsenic, cadmium, copper, lead, and zinc in water bodies have recently been revealed in innumerable countries (Kim *et al*., 2020). In aquatic systems, arsenic mostly exists in two oxidation states, arsenite (As (III)) and arsenate (As (V)), depending upon pH and redox demands. The toxicity and transportability of arsenic are influenced by its oxidation state; As (III) is known to be more toxic than As (V) (Sharma *et al*., 2009). It emanates from both natural sources including the weathering and/or disintegration of As-bearing minerals such as arsenopyrite and orpiment as well as anthropogenic origins such as mining activity, pesticides, and wood chemical preserving agents (An *et al.,* 2020).

The new scientific survey proposes the relationship of oxidative stress in the pathogenesis of atherosclerosis, inflammation, cancer, and neurodegenerative disorders like alzheimer, Parkinsonism together with convulscent. The naturally existing heavy metal arsenic is an omnipresent environmental pollutant with demonstrated adult toxicity in humans and teratogenicity in both laboratory animals and humans (Mukhopadhyay *et al*., 2019). Oxidative stress is principally brought about by the reactive oxygen species. This species consists of both oxygen radical such as peroxyl, superoxide anion, hydroxyl, peroxynitrite along with nitric oxide radical and non-radical derivatives of oxygen like hydrogen peroxide, hypochlorous acid, as well as singlet oxygen (Ramani *et al*., 2012). The flavonoids are common phenolic compounds and plentiful in plants. The prevailing feature of flavonoids is hydroxyl group replaced flavan moiety. These unique chemical functionalities are indispensable for the flavonoids to scavenge free radical and rule out the oxidation of biological molecules by

transforming the more reactive oxygen species by donating hydrogen into inactive species in systems (Middleton *et al*., 2000). *Leucas lavundulifolia* has been largely employed in cattle and human maladies such as a cough, fever, and headache, loss of appetites, cold, skin diseases, snake bites, scorpion sting, and migraine. The aerial part of this plant has been used as a sedative, laxative, Jaundice, expectorant, stomachic, dyspepsia, antihelminthic, stomachic, scabies, psoriasis, migraine, glaucoma, asthma, and paralysis (Islam *et al*., 2017)

In the current study sodium arsenate (V) induced cytotoxicity along with genotoxicity was studied in intestinal epithelial cells. The crude extract was tested for flavonoids and phenolic compounds through DPPH and ABTS free radical scavenging assay. ROS, MMP, reduced chromatin condensation, SOD and DNA damage were studied in intestinal epithelial cells.

MATERIALS AND METHODS

DPPH assay

Free radical scavenging assay was carried out to estimate the antioxidant capacity of the extract. DPPH free radical scavenging assay was used to evaluate the free radical scavenging capability of the crude extract. 1 mL of 0.1 mM DPPH solution prepared in methanol was mixed with 1.0 mL of the extract varying concentration (7.8, 15.6, 31.25, 62.5, 125, 250, 500) µg/mL, after 20 min optical density was read at 517 nm with the help of photometer (Bansal *et al*., 2011).

ABTS assay

ABTS free radical scavenging assay was carried out to evaluate the antioxidant capacity. ABTS (7 mM) solution was taken along with 2.4 mM potassium per sulphate, this reaction mix was kept in dark for 15 hours. Flower extract was dissolved in methanol. 180 mL of ABTS was mixed with the 20 mL of test solution. The reaction mixture was allowed to stand for 20 min at room temperature. Absorbance was recorded at 750 nm in a photometer (Tachakittirungrod *et al*., 2007).

Cell culture

HepG2 cells were procured from NCCS, India. Epithelial cells were grown in culture flask (Falcon, USA), DMEM (High glucose) with FBS 10%, penicillin–streptomycin 1%, at 37 °C in a CO_2 incubator (NuAire, Plymouth, USA) with 5% CO_2. For various assays, epithelial cells were maintained at 85% confluence in T- 25 culture flasks and utilized. Viability of the cells were assessed with the help try pan blue dye exclusion assay.

MTT assay

MTT assay was carried out to determine the cytotoxicity of the arsenate. Cells were allowed to attain 85-90% confluence then used for the assay. Epithelial cells were trypsinized, harvested and seeded about 1x 10^4 cells per well. Adhered cells were treated with various concentration of sodium arsenate (2.5, 5, 10, 25, 50, and 100) µM, flower extract and sodium arsenate in combination with crude extract to assess the cytotoxicity. At the end of the experiment formazancrystals formed were dissolved in 0.1 mL of DMSO and optical density was read at 550 nm using the photometer (Sladowski *et al*., 1993).

Estimation of reactive oxygen species (ROS)

The reactive oxygen species concentration was measured using flow cytometry. Cells were placed at a density of 3x 10^5 in small petri dishes and were treated with sodium arsenate, extractand blend of extract along with arsenate. The cultures were kept for an hour (Eruslanov and Kusmartsev, 2010). The medium was decanted post incubation, and 5 µM DCFDA was added. Later kept for another 30 min in 5% CO_2 incubator at 37 °C. Epithelialcells were suspended in PBS after harvesting, flow cytometer (Becton Dickinson, USA) was used for analysis.

Evaluation of membrane potential of mitochondria ($\Delta\psi m$)

To estimate MMP, Epithelial cells were placed in 6 cm^2 Petri dishes at a density of 3x10^5. Cells were kept for 24h with sodium arsenate, crude extract and the blend of both. After adding fresh medium, Rhodamine 123 (5 µg/mL) was added. The cells were kept at 37 °C for 30 min in 5% CO_2 incubator. Trypsinised cells were harvested, washed with phosphate buffer and centrifuged (1000 rpm) for 10 min (Scaduto Jr *et al*., 1999). Cellswere harvested in cold PBS, FACS Calibur (Becton Dickinson, USA) was used to acquire the information, after suspending the cells in 1 mL Chilled PBS.

Comet assay

DNA breaks (Single strand) were calculated by single cell gel electrophoresis. Epithelial cells were

scattered at a frequency of 7×10^5 cells in 6 cm^2 plates. Epithelial Cells were administered with sodium arsenate, extract, and incubated for 24 h. After incubation further washed and collected with PBS and suspended in low melting agarose. Approximately $2x10^4$ cells were suspended in low melting point agarose. Agarose covered slides were layered with low melting agarose along with cells maintained at 37 °C and recoated with ordinary melting agarose. The slides were immersed in the lysing solution maintained at 4 °C overnight; slides were transferred to an electrophoretic chamber containing an alkaline buffer (pH 13). The lysed cells were subjected to electrophoresis in a horizontal tank for 26 min (300mA, 20V). The slides were washed with neutralizing buffer. Ethidium bromide-stained slides were immediately observed under the fluorescence microscope photographed and analyzed with software (Singh *et al.*, 1988).

Evaluation of nuclear condensation

Epithelial cells were distributed in a 6 well plate at a frequency of 3×10^5 per well, then preserved overnight in a CO_2 incubator at 37 °C, and then treated to above mentioned combinations. 0.3 mL of EtBr and DAPI was added to each well then the plate was kept at 37 °C for 30 min. At the end, cells were washed with PBS and observed under the fluorescence microscope for fragmentation and nuclear condensation (Renvoize *et al.*, 1998).

RESULTS AND DISCUSSION

DPPH and ABTS free radical scavenging assay were performed with *Leucas lavundulifolia* crude extract. α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. This is the simplest method, wherein the prospective compound or extract is mixed with DPPH solution and absorbance is recorded after a defined period (Kedare *et al.*, 2011). DPPH radical scavenging activity was found to be 86.22 ± 3.2 (IC_{50} µg/mL). Decoloration method is capable of determining both hydrophilic (in buffered media) and lipophilic (in organic media) antioxidant properties in complex samples (Cano *et al.*, 2002). ABTS radical scavenging activity was found to be 67.20 ± 1.8 (IC_{50} µg/mL). Both assays carried out prove the presence of antioxidant activity in the *Leucas lavundulifolia* extract.

Aerial part of the plant is used as functional food in some of the geographical regions. Antioxidant activity can be assigned to flavonoids and polyphenols in the extract. Polyphenols are secondary metabolites found in plants. They consist of phenols, phenolic acids, quinones, flavonoids, tannins, together with phenylpropanoids (Derouich *et al.*, 2020).

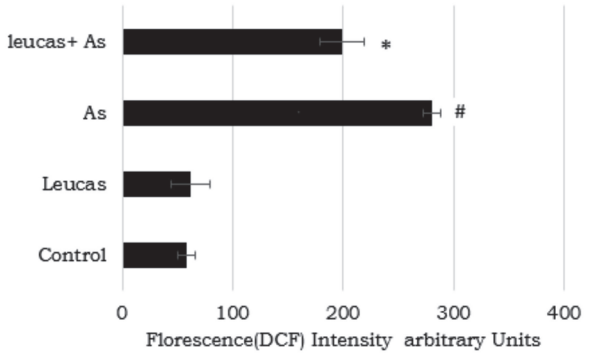

Fig. 1. *Leucas lavundulifolia* extract decreases arsenate induced ROS in epithelial cells. Results are significant ($p<0.05$). * Extract pre-treatment compared to arsenate treated cells. # Arsenate compared to control cells.

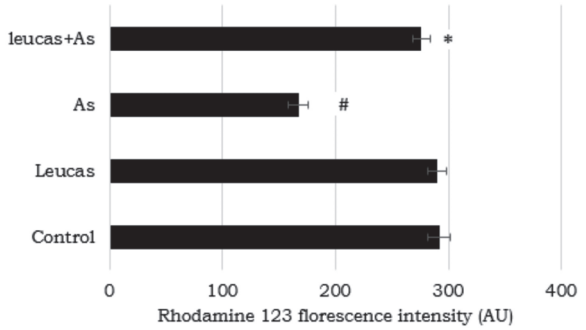

Fig. 2. Crude extract of *Leucas lavundulifolia* increases MMP in epithelial cells compared to arsenate treated cells.* Extract pre-treatment compared to arsenate treated cells. # Arsenate compared to control cells.

Table 1. DPPH and ABTS assay

Tests	*Leucas Lavundulifolia*	Ascorbic acid (control)
DPPH free radical scavenging assay (IC_{50} µg/mL)	86.22 ± 3.2	7.12±0.32
ABTS free radical scavenging assay (IC_{50} µg/mL)	67.20 ± 1.8	2.15±0.9

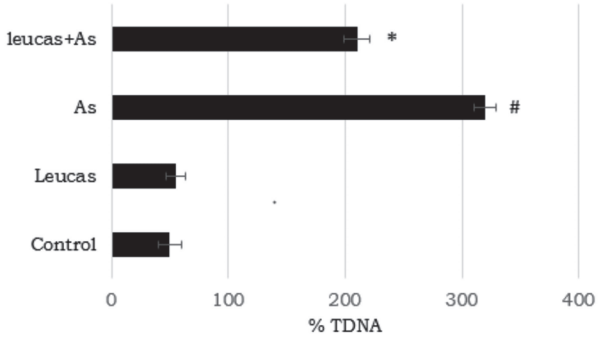

Fig. 3. *Leucas lavundulifolia* extract reduced occurrence of % TDNA in epithelial cells. *Extract pre-treatment compared to arsenate treated cells. # Arsenate compared to control cells.

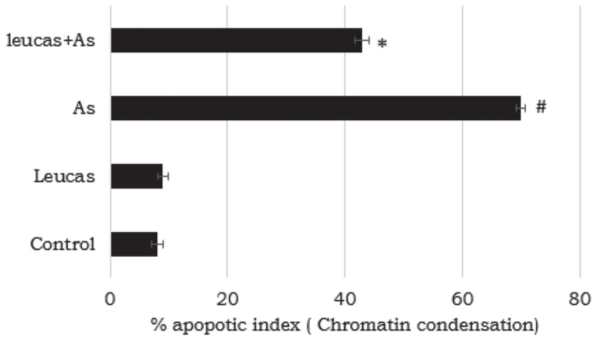

Fig. 4. Reduction in chromatin condensation in *Leucas lavundulifolia* extract treated epithelial cells. *Extract pre-treatment compared to arsenate treated cells. # Arsenate compared to control cells.

Reactive oxygen species (ROS) play a myriad of signalling roles in diverse organisms from bacteria to mammalian cells. As a faction of chemical species that consist of at least one oxygen atom in individual molecule but exhibit firmer reactivity than molecular oxygen, ROS involves free radicals like superoxide, hydroxyl radical, as well as singlet oxygen, and nonradical species such as hydrogen peroxide formed by the partial reduction of oxygen (Zhang *et al*., 2016). Arsenic is known to induce ROS in mammals. Excess ROS will lead to cellular damage at various levels. Current study showed increase in ROS levels when subjected to sodium arsenate. *Leucas lavundulifolia* crude extract pre-treatment decreased ROS levels significantly in intestinal epithelial cells ($p<0.05$). The mitochondrial membrane potential ($\Delta\psi m$) developed by proton pumps (Complexes I, III and IV) is a necessary constituent in the course of energy repository during oxidative phosphorylation. Simultaneously with the proton gradient (ΔpH), $\Delta\psi m$ composes the transmembrane potential of hydrogen ions which is mobilized to make ATP. The degrees of $\Delta\psi m$ along with ATP in the cell are kept comparatively stable although there are limited variations of both these components that can occur speculating normal physiological activity (Zorova *et al*., 2018). *Leucas* extract which is also known functional could decrease the altered membrane potential in epithelial cells significantly indicating the mitoprotective effect ($p<0.05$).

Arsenic exposure induces DNA damage via oxidative stress in well-known fact (Tran *et al*., 2002). Pre-treatment of epithelial cells with crude extract quenched ROS efficiently as a result lesser DNA damage was found in culture cells. % T DNA decreased significantly in test groups compare to the negative controls ($p<0.05$). Nuclear condensation and cytoplasmic shrinkage is a phenomenon explaining free radical activity and excess peroxide. Dehydration is also reason for this. The administration of crude extract decreased the nuclear condensation and cytoplasmic shrinkage significantly in epithelial cells ($p<0.05$).

CONCLUSION

Leucas lavundulifolia is a known functional food in Asiatic regions. The free radical scavenging assays could prove the presence of the antioxidants. The flavonoids and phenolic compounds present in the extract helped in quenching reactive oxygen species. It also showed mitoprotective ability. This plant was explored for its potential to alleviate arsenic induced cellular dysfunctions in epithelial cells. The crude extract was efficient in harmonising the effect at molecular level. Further work need to be done in order to see DNA repair and other aspects.

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