Amelioration of methylmercury induced neural damage by essential oil of *Selinum vaginatum* (Edgew) C. B. Clarke

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Abstract: Methylmercury (MeHg), an organometallic contaminant is a well spotted cause for a series of disorders, especially in the central nervous system. As there is no proper treatment, *Selinum vaginatum* (Edgew) C. B. Clarke, a traditional medicinal plant, is taken in the present study for assessing its neuroprotective effect against MeHg induced toxicity using rat brain mitochondrial fractions. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide dye (MTT) assay indicated that there was a reduction in the mitochondrial viability in MeHg treated sample and IC_{50} value recorded was 2.5µg/ml. Biochemical analysis showed that there was a significant inhibition of glutathione levels (GSH) and catalase activity and an elevation of thiobarbituric acid reactive substances (TBARS) levels in MeHg treated sample. These changes were prevented by co-incubation with essential oil extracted from *Selinum vaginatum*. The GSH reduction caused by MeHg is restored by essential oil, endorsing its chelating effect, an important molecular mechanism of defense against oxidative injury. Some of the major compounds are detected in Gas chromatography-mass spectrometry (GC-MS) analysis of essential oil, which could be accountable for its neuroprotection against MeHg.

Keywords: Methylmercury, Essential oil, Neuroprotection, *Selinum vaginatum*, Oxidative stress.

INTRODUCTION

Methylmercury (MeHg) is an environmental toxicant that established neural damage both in humans and experimental models (Clarkson *et al.,* 2003). The most affected system due to MeHg toxicity is central nervous system (CNS) that leads to neurodegenerative disorders (Aschner *et al.,* 2007; Sakaue *et al.,* 2006). MeHg poisoning is predominant in the communities, which are mostly depending on fish for food as sea water is contaminated with MeHg due to anthropogenic sources (Clarkson, 2002; Clarkson *et al.,* 2003). MeHg-induced oxidative stress diminishes non-enzymatic antioxidant like GSH, and enzymatic antioxidants such as catalase, superoxide dismutase etc. whereas boosts the levels of peroxides and TBARS (Farina *et al.,* 2005; Franco *et al.,* 2006). The use of chelating agents to counteract MeHg poisoning has several disadvantages due to their side effects.

Therefore, the major research focus is mainly on exploration of natural potential neuroprotective agents from plant sources against MeHg induced toxicity. One of such plant is *Selinum vaginatum* (Edgew) C.B. Clarke (Umbelliferae), also traditionally known as Bhootkeshi, an indigenous drug found in Himalayan region. The roots and the rhizomes of *S. vaginatum* have been used to treat neurological disorders, insomnia, epilepsy, hysteria, convulsions etc. It has been reported to have antioxidant and lipid per oxidation functions (Pandey *et al.,* 2013; Rao *et al.,* 2005). *S. vaginatum* has been employed traditionally for handling neurological disorders because of its antioxidant property (Pandey *et al.,* 2013; Chauhan *et al.,* 2012). Hence, in the present study, an attempt was made to determine the neuroprotective potential of the essential oil extracted from *S. vaginatum* roots against MeHg using rat brain mitochondrial fractions.

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MATERIALS AND METHODS

Animal

Adult Wistar rat (9 month old) were bred in the animal house of VIT University, Vellore. All experiments were approved by the institutional animal ethics committee (VIT/IAEC/VIIth/14). Animal were maintained at 23°C on 12h light/dark cycle with free access to water and food (VIT University, Vellore, India).

Chemicals

Methyl mercury (II) chloride, DTNB, GSH reduced form and MTT were purchased from sigma Aldrich (St.Louis, MO). All other chemicals used were of analytical grade.

Plant sample

Roots of *Selinum vaginatum* (Edgew) C. B. Clarke were collected in October 2013, from Tungnath, Rudraprayag, Uttarakhand. A voucher specimen GUH 1281 was deposited and identified by Prof. P Prasad, High altitude Plant Physiology Research Center, Hemwati Nandan Bahuguna Garhwal University.

Extraction of essential oil by hydro distillation method

The plant material (87g of roots) was washed with tap water and air-dried. It was then placed in round-bottom

flask and then 500ml of distilled water was added to it. Hydro distillation was carried out for 15 hours at 100°C using the Clevenger–type apparatus (Chauhan *et al.,* 2012).

Isolation of mitochondria from rat brain

Mitochondrial enriched fractions were prepared as per the procedure described by (Andersen *et al.,* 2003). A pellet containing synaptosomes and free mitochondria along with a myelin-rich supernatant was obtained. The mitochondrial fractions were kept on the ice for 10-15 min.

Protein estimation

Concentration of protein in the mitochondrial enriched fractions was estimated using bovine serum albumin (BSA) as a standard (Lowry *et al.,* 1951)

Incubations

Approximately 1.6mg of protein containing pellet was incubated for 30min at 25°C with different concentrations of the essential oil of *Selinum vaginatum* (0.5, 1.0, 1.5 and 2.0µg/mL) dissolved in 0.1% DMSO and MeHg (1.2, 2.5, 6.25, 12.5, 18.5, 25µg/ml) making the incubation volume to 150µL. After the incubation period, the following parameters such as mitochondrial cell viability, catalase activity and glutathione and TBARS levels were assessed.

MTT cell viability assay

Mitochondrial function was assessed by using MTT (InSug *et al.,* 1997). 150µL of 0.6mM MTT was added to the 150µL preincubated reaction medium containing different concentrations of essential oil of *S. vaginatum* and/or different concentrations of MeHg for 30 min at 25°C. The color of formazan crystals formed was measured at 550nm and was expressed in terms of percentage of viability.

Glutathione (GSH) assay

GSH content was measured by the method of Ellman, 1958. Ellman's reagent was added to the preincubated reaction medium. The absorbance was taken at 650 nm and was expressed in terms of percentage of control.

Catalase activity

Catalase activity of the essential oil was determined by the method of Sinha, 1972. To the pre incubated reaction medium, 0.2 M H_2O_2, 5% Dichromate and acetic acid mixture (1:3) was added and then incubated for 10 min at 90πC in water bath. The absorbance was taken at 620 nm and was expressed in terms of percentage of control.

TBARS assay

TBARS assay was performed to determine lipid peroxidation (Ohkawa, 1979). To the pre incubated reaction medium, 0.375% 2-thiobarbituric acid, 5% trichloroacetic acid, and 0.25N HCl were added in equal volume and incubated for 1 hour at 95°C. The absorbance was taken at 535 nm and was expressed in terms of percentage of control.

Chelating activity

Chelating activity was done as per the method of Jeferson *et al.,* 2007. MeHg (2.5µg/ml) was incubated with GSH (100µM) in the presence/absence of the essential oil of *S. vaginatum* (2µg/mL) at 25°C for 30 min. After the incubation period, the amount of GSH was determined with the addition of 5, 5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1958).

Identification of components of essential oil – GC/MS

The composition of essential oil was analyzed on a Perkin Elmer gas chromatograph Model clarus 680, which is coupled to a Perkin Elmer MS Model clarus 600 equipped with an Elite-5 MS column (30.0m × 0.25mm; 250µm); with initial temperature of 60°C (2min) to 300°C at 10°C/min and a 6min hold. As carrier gas helium was used (1.0mL/min); injection was in split mode (10:1), injector temperature was 250°C. The MS was operated in the Electron Impact mode at 70 eV and the electron multiplier voltage was 1200 V. The temperature of ion source was 240°C. In the scan mode in the *m/z* range of 50-600, mass spectra data were obtained. The essential oil of *S. vaginatum* was diluted in hexane at 1:30 ratio (Rasheed *et al.,* 2010; Seshadri *et al.,* 1967; Chauhan, 1999).

STATISTICAL ANALYSIS

Using one-way analysis of variance, statistical differences among the groups were analyzed. Statistically significant differences were considered when $P<0.05$ and all the experiments were done in triplicates.

RESULTS

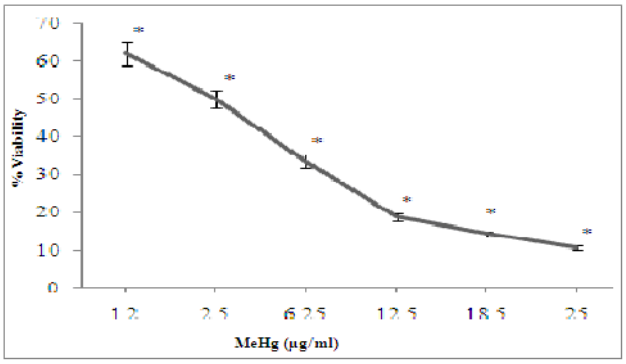
MTT cell viability assay

MeHg decreased mitochondrial viability in MTT assay and IC_{50} value reported was 2.5µg/ml (fig. 1). The effect of essential oil of *S. vaginatum* on cell viability on mitochondria of rat brain is shown in fig 2. Treatment of cell with MeHg altogether confirmed cytotoxicity and it is inferred that essential oil extracted from *S. vaginatum* protects the cell even at low concentration (0.5µg/mL of essential oil of *S. vaginatum* exhibited 73% cell viability). With an increase in the concentration, it showed higher protection to the cell against MeHg.

Glutathione (GSH) assay

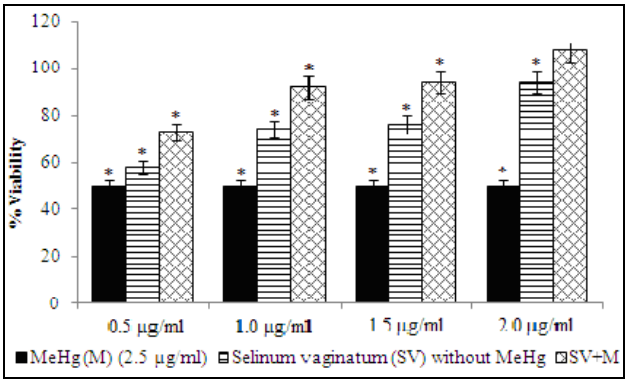
The impact of essential oil of *S. vaginatum* on GSH level of the cells has been demonstrated in fig. 3. It can be seen from the results that the level of glutathione is decreased in MeHg treated fraction whereas essential oil of *S. vaginatum* completely restored the GSH levels when

added to the MeHg induced rat brain mitochondrial fractions. Essential oil of *S. vaginatum* (1.5µg/mL) exhibited 94% GSH level.



Data is expressed as Mean ± Standard Error and is represented as % Viability *indicates statistically different from control (P<0.05)

Fig. 1: Effect of MeHg on mitochondrial viability



Data is expressed as Mean ± Standard Error and is represented as % Viability *indicates statistically different from control (P<0.05)

Fig. 2: Effect of essential oil of *S. vaginatum* on mitochondrial viability.

Catalase activity

Outcome of catalase specific activity of the essential oil of *S. vaginatum* is indicated in fig. 4. From the fig. it might be derived that at low concentration i.e. 0.5µg/mL of essential oil of *S. vaginatum* showed catalase specific activity but at concentration of 1.5µg/mL of essential oil exhibited generally higher catalase specific activity i.e. 101%.

Thiobarbituric acid reactive substances (TBARS) Assay

The capability of the essential oil of *S. vaginatum* to protect the lipids from oxidation has been indicated in fig. 5. It can be inferred from the results that essential oil of *S. vaginatum* demonstrated protection against lipid per oxidation. The outcome demonstrated that the level of protection against lipid per oxidation was concentration dependent. 1.5µg/mL of essential oil of *S. vaginatum* exhibited higher protection than at lower concentration.

Chelating activity

Essential oil of *S. vaginatum* diminished GSH oxidation brought about by MeHg affirming its chelating activity, which is also one of the molecular mechanisms of protection against oxidative damage. The chelating activity of *S. vaginatum* is show in table 1. The results showed that 100 µmoles of reduced glutathione used in the study was decreased in the presence of mercury i.e. 50.15±1.3 µmoles whereas, this value is increased to some extent in the presence of essential oil i.e. 59.9±4.2 µmoles that shows the chelating ability of essential oil. The presence of essential oil in the reaction medium changed the oxidative capability of MeHg that converts reduced glutathione to oxidized form.

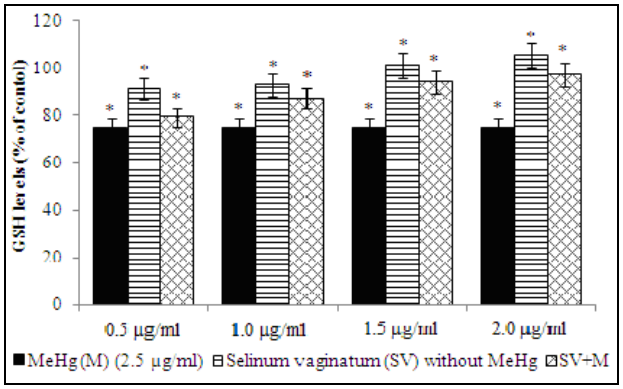


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Fig. 3: Effect of essential oil of *S. vaginatum* on glutathione oxidation.

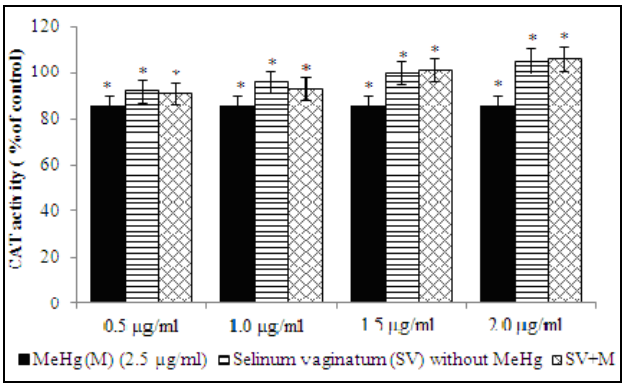

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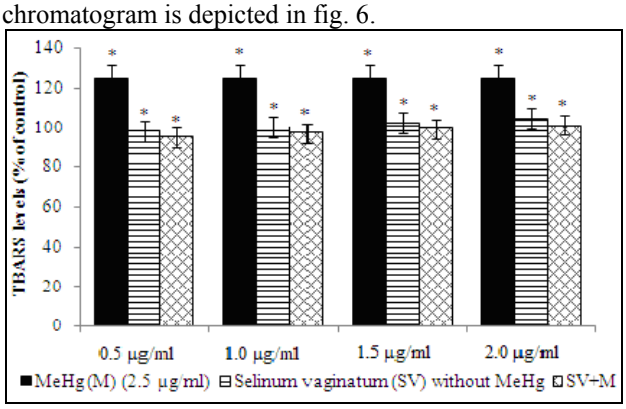
Fig. 4: Effect of essential oil of *S. vaginatum* on catalase activity.

GC-MS Analysis

A total of 11 constituents of 66.71% of the total volatile parts were identified with GC-MS. Details of the identified compounds are presented in the table 2. Retention time, Kovats index and percentage of each compound are included in the table. The major

components of the essential oil was 2-Propenoic acid (20.12%), Patchouli alcohol (11.48%), Isocyclocitral (10. 42%), whereas a few other important constituents, e.g., Longifolenaldehyde (6.27%), β-Phellandrene (4.97%), etc were also present in considerable amounts. GC-MS chromatogram is depicted in fig. 6.



Data is expressed as Mean ± Standard Error and is represented as % Viability *indicates statistically different from control (P<0.05)

Fig. 5: Effect of essential oil of *S. vaginatum* on lipid per oxidation.

Table 1: Chelating activity of *S. vaginatum*

	Levels of GSH (µmole)	
	Without *S. vaginatum* (2µg/mL)	With *S. vaginatum* (2µg/mL)
GSH without MeHG	100±0.0	95.95±4.8
GSH with MeHg (2.5µg/ml)	50.15±1.3	59.9±4.2

DISCUSSION

In the present investigation, essential oil extracted from *S. vaginatum* was used in the isolated rat brain mitochondrial fractions to investigate its neuroprotective effect against MeHg toxicity. Several studies reported that oxidative stress as the major mechanism due to MeHg cytotoxicity that alters the mitochondrial membrane potential, that in turn leads to a mitochondrial burst of reactive oxygen species (ROS) production (Kim and Sharma, 2003; Dreiem and Seegal, 2007).

In this respect, mitochondria play a vital role for assessing the neuroprotective potential of medicinal plants. To the best of our knowledge, there have been no reports on the *in-vitro* study of essential oil extracted from *S. vaginatum* and its protective effect against MeHg induced toxicity. The effect of MeHg and essential oil on mitochondrial viability was evaluated by measuring MTT reduction. From the data it was inferred that exposure of MeHg decreases mitochondrial viability whereas, essential oil of *S. vaginatum* at lowest concentration used in the present study ((0.5µg/mL) showed higher protection (73% viability) to the cell against MeHg induced toxicity.

Table 2: Composition of Essential Oils of Roots of *Selinum vaginatum*

Compound	RT	KI	%	Reference
α-Pinene	4.654	431	1.670	Chauhan, 1991
Camphene	4.929	486	0.430	
β-Phellandrene	6.255	751	4.974	
2-Propenoic Acid	10.112	1522	20.120	
Naphthalene	11.412	1782	0.982	
Azulene	13.008	2101	3.918	
Carotol	14.283	2356	4.653	
Isocyclocitral	14.789	2459	1.789	
Isocyclocitral	14.793	2458	10.425	
Patchouli Alcohol	15.129	2525	11.480	
Longifolenaldeyde	15.454	2590	6.275	

RT – Retention Time, KI – Kovats Index, % - Area Percentage

However, different *in vivo* and *in vitro* trial observations have shown that the toxic effects of MeHg are followed by a significant reduction of GSH (Glaser *et al.,* 2010; Farina *et al.,* 2005). The knowledge about the antioxidant capacity of the plant was established when the GSH level in the cells increased on treatment with the essential oil. Catalase, which is one of the intracellular antioxidative enzymes, plays a central role in the defense mechanisms against MeHg-induced oxidative damage (Nita *et al.,* 2001; Islekel *et al.,* 1999). Reduction of this enzyme is seen in damaged mitochondrial cells. Therefore, to determine the neuroprotective activity of the essential oil of *S vaginatum*, catalase assay was carried out. It was observed that 1.5µg/mL of *S. vaginatum* exhibited higher catalase specific activity. Lipid peroxidation and its reactive products can affect the important cellular components, leading to cytotoxicity (Jia and Misra, 2007). Our results showed that MeHg exposure increased the levels of TBARS, conversely essential oil diminishes TBARS, confirming its neuroprotective potential. Similar findings were observed by our previous study (Dhanoop et al., 2015; Dhanoop et al. 2015a) as well as by Glaser *et al.,* 2010; Franco *et al.,* 2006; Farina *et al.,* 2005. Chelating effect of essential oil was also confirmed by its ability to diminish MeHG induced GSH oxidation. This ability may be due to the presence of several volatile components present in the essential oil of *S. vaginatum* that was detected by GC-MS.

CONCLUSION

From the study, it was shown that the essential oil extracted from *S. vaginatum* at 1.5µg/mL concentration exerts potential neuroprotection against MeHg induced toxicity in rat brain mitochondrial fractions. These observations suggest that *S. vaginatum* proves to be a promising neuroprotectant in the prevention of several neurological disorders.

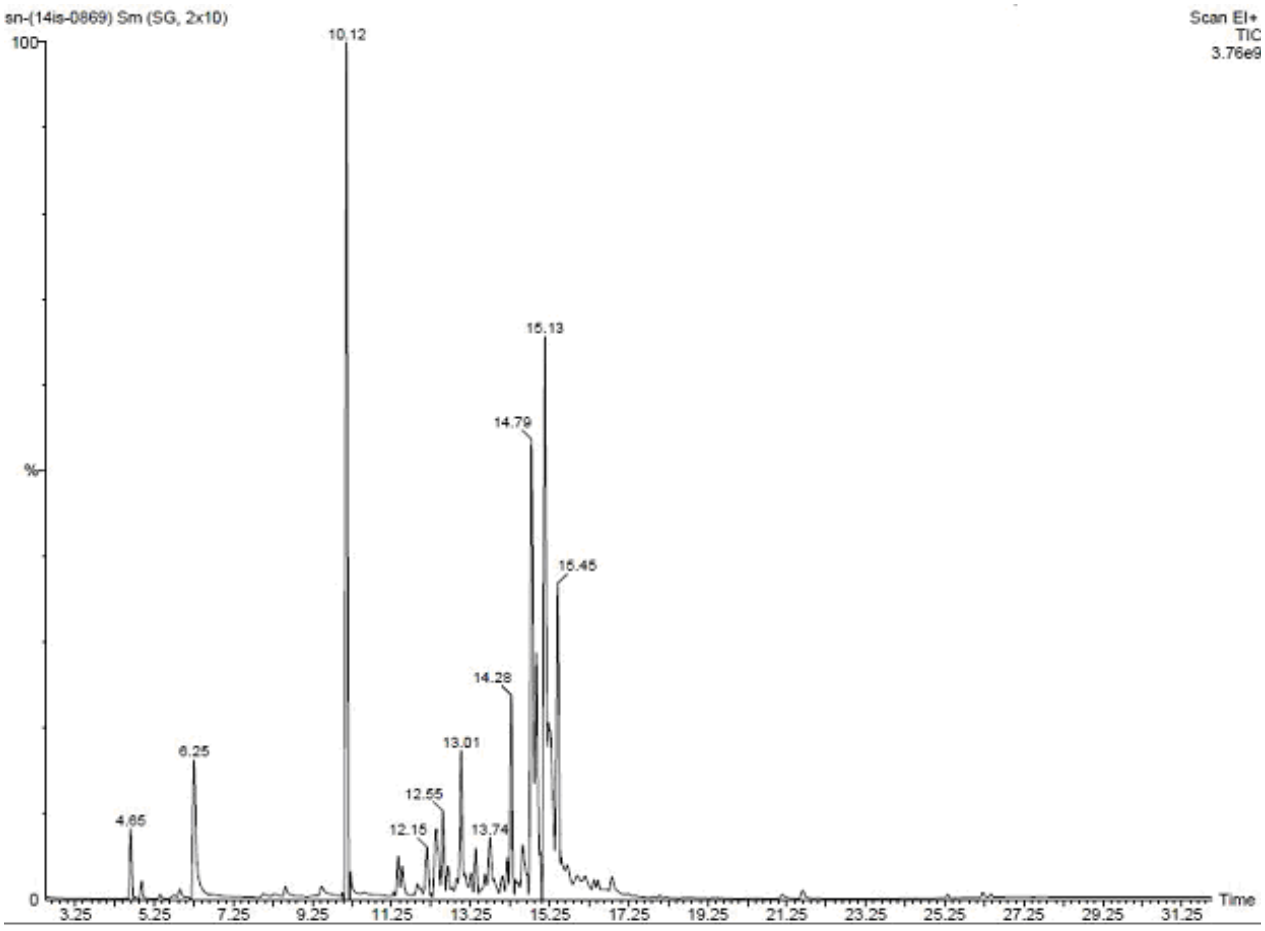


Fig. 6: GC-MS Chromatogram of essential oil of *S. vaginatum*.

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