Research Article

Alteration in Antioxidant Enzyme Activities by Nickel-Induced Hepatotoxicity in Wistar Rats: Ameliorating Effect of the Aqueous Extract of *Myristica fragrans* (nutmeg)

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ABSTRACT: The ameliorating effect of *Myristica fragrans* (nutmeg) on lipid peroxidation (LOP), reduced glutathione (GSH), glutathione-s-transferaes (GST), glutathione peroxidase (GPx), Catalase (CAT), Superoxide dismutase (SOD) as well as some liver marker enzymes were examined using a model of $NiCl_2$–induced oxidative stress in rats. Thirty male Wistar rats were used in this study. They were randomized into 5 groups of six rats per group. Animals in group I received distilled water and normal rat feed only. Rats in groups II and III received only 0.75 mg/kg/day and 1.5 mg/kg/day of $NiCl_2$ respectively. All rats in groups IV and V were pretreated with 200 mg/kg body weight and 300mg/kg body weight of the aqueous extract of *Myristica fragrans* (AEMF) respectively. In addition to the doses of the extracts administered to the above groups, group IV rats received 0.75 mg/kg/day of $NiCl_2$ while group V animals were treated with 1.5 mg/kg/day of $NiCl_2$. All administration was carried out by gastric intubation for 30days. $NiCl_2$ caused significant increase ($p<0.05$) in the level of MDA formation in the liver, suggesting pro-oxidant induced membrane damage. These were followed by a significant decrease ($p<0.05$) in the levels of GSH, GPx, GST, SOD and CAT. The aqueous extract of *Myristica fragrans* (AEMF) in a dose related manner, significantly ($p<0.05$) reduce the level of MDA as well as the antioxidant markers to a near constant value when compared with the control. Pretreatment with AEMF also led to a significant decrease ($p<0.05$) in liver marker enzymes (ALT, ALP, AST, LDH, GGT and CK) when compared with the $NiCl_2$ treated rats in a dose-dependent manner. Upon the administration of AEMF, histological examination did not reveal much pathophysiological changes when compared with the control.

KEYWORDS: Antioxidant enzymes, lipid peroxidation, liver marker enzymes, $NiCl_2$ and *Myristic fragrans* (AEMF)

INTRODUCTION

Nickel is a hard, ductile and malleable element that belongs to the group of metals in the periodic table called the transitional metals. Like selenium, zinc, iron, iodine e.t.c., there is evidence that nickel might be an essential trace element (Goyer, 1991) probably because studies have indicated that nickel is needed in trace amount as a cofactor in the absorption of iron from the intestine under certain conditions (Das *et al*., 2008). Nickel primarily coexists with oxygen or sulphur forming oxides or sulphides that occur in the earth crust naturally (Das *et al*.,2008).

The properties of nickel and its distribution in the environs have put it in the same class by the Agency for Toxic Substances and Disease Registry (ATSDR) as a toxic substance as other heavy metals (ATSDR, 1988). The generation and release of nickel into the atmosphere as well as increased deposition in water bodies have caused adverse effects to the health of living organisms including humans. Such adverse effects of nickel is dependent on the route of exposure and the solubility of the compound (Coogan *et al*., 1989). The toxicity of nickel to humans and other animals is due to the formation of free radicals which causes various DNA modifications, increase lipid peroxidation as well as glutathione depletion (Das *et al*, 2008; Esterbauer *et al*, 1991). The balance between free radical generation and its removal, quenching and scavenging determines the susceptibility of a cell to oxidative damage. Although organisms possess antioxidants defense systems, these protective mechanism are insufficient in handling cumulative damage caused by endogenous and exogenous oxidants (Simic, 1988; Sun, 1990). In this point of view, much attention has been targeted on the protective biochemical mechanisms of naturally occurring antioxidants in biological systems and their mode of action (Usoh *et al*., 2005). In most tropical regions of Africa, the recognition and reliance on herbs and spices as food and as medicine has being on the increase. The search for numerous plants as useful sources of exogenous antioxidants has generated much concern in the food industry as well as in folk medicine. One of such plant is the dried nut seed of *Myristica fragrans* belonging to the family of Myristicaceae with an active ingredient called Myristicine (Lee *et al*., 2005). In Nigeria, *Myristica fragrans* commonly known as nutmeg has being channeled into diverse uses. The plant is widely used locally to spice meals and also as flavoring agents for baked foods and for some vegetable soup preparations. Eweka and Eweka (2010) reported that nutmeg and its oleoresin have been used in the preparation of meat products, soaps, sauces, baked foods, confectioneries, puddings, seasoning of meat and vegetables, to flavor milk dishes and punches. Studies have shown that the aqueous extract of nutmeg possesses significant potential as hepatoprotective and antioxidative agent against ISO-induced damage in rats (Mohammed *et al*., 2013) and has being used at a dose of 500 mg/kg as aphrodisiac and psychoactive agent in male rat (Tajuddin *et al*., 2003, Tajuddin *et al*., 2005). This present research

attempts to evaluate and corroborate the antioxidant properties of the aqueous extract of *Myristica fragrans* on some biomarkers of oxidative stress as well as enzyme markers of the liver in Wistar rats treated with nickel chloride ($NiCl_2$).

MATERIALS AND METHODS

Plant material

Fresh *Myristica fragrans* (nutmeg) was purchased from the Main Market, Abraka, delta State, Nigeria. The *Myristica fragrans* nuts were authenticated at the herbarium of the Department of Botany, Delta State University, Abraka, Nigeria, where some specimens were deposited for future references. This research study commenced between the periods of September through November 2015.

Myristica fragrans preparation

The preparation of *Myristica fragrans* extract was carried out in accordance to the method described by Mohammed *et al*. (2013). The fresh *Myristica fragrans* (nutmeg) was air dried for about a three to four weeks and made into power by using an electric blender. The powdery substance obtained from the spices was reweighed. A 500 g sample of the powdered *Myristica fragrans* was boiled in distilled water. The mixture was filtered using a Whatmann No. 40 filter paper, and evaporated to dryness by slow heating and continuous stirring of the extract in a water bath at 40-50 ºC. The brownish residue obtained was collected and used for the study.

Ethical consideration

Ethical conditions guiding the conduct of experimental animals were observed as stipulated by Ward and Elsea (1997) and all NIH Guide and Use of Laboratory Animals. The experimental protocol was approved for the use of laboratory animals by the ethical committee of the Faculty of Basic Medical Sciences, Delta state University, Abraka, Delta State, Nigeria.

Toxicity Test and LD_{50} Study

The aqueous extract of *Myristica fragrans* at different dose concentrations (200−2000 mg/kg) were administered to four groups of 5 rats each to determine the LD_{50} according to the method of Lorke (1983). The Wistar rats were deprived meal for 24 hours prior to the start of the study, but were granted free access to water. The animals were observed for toxic symptoms such as hyperactivity, convulsions, behavioural changes, and mortality for 72 hours. Groups that will cause 50% death were noted.

Experimental Animals and Design

Thirty adult male rats of Wistar strain weighing between 120−150 g were purchased from College of Medical Science, Ambrose Ali University, Ekpomah, Edo State. The animals were allowed to acclimatize to the laboratory condition of

temperature 25±2 °C and 12-h light/dark cycle for two weeks at the Animal House, Basic Medical Sciences, Delta state University, Abraka. All rats were allowed free access to feed. The feed products were obtained from Edo Feed and Flour Mill (BFFM), Ewu, Edo State, Nigeria and drinking water *ad libitum*.

The animals were divided into five groups each of six rats per group. Group I (control) neither received $NiCl_2$ nor the extracts. Control rats were fed with standard rodent pellet diet and received distilled water only over a period of 30days. Group II and III animals were treated with 0.75 mg/kg/day and 1.5 mg/kg/day of $NiCl_2$ only, once for a period of 30 days. Also, animals in Groups IV received 0.75 mg/kg/day and were treated with 200 mg/kg of *Myristica fragrans* daily for 30 days. Finally, Group V animals were administered 1.5 mg/kg/day of $NiCl_2$ and treated with an increased dose of 300mg/kg of *Myristica fragrans* for the same duration and in the same manner.

The protocol of administration of $NiCl_2$ dose used in this study was adapted from Weischer *et al*. (1980). Administration of the extract (*Myristica fragrans*) was done by oral intubation once daily for days of the experimental periods.

Preparation and collection of plasma and tissues homogenate

After 30 days of the experimental period, rats from each group were fasted overnight and were sacrificed by cervical dislocation between the hours of 6 am and 9 am. Blood was collected via heart puncture by means of a 5 ml hypodermic syringe and placed in ice-cold tube containing heparin. The blood sample was centrifuged at 3000 rpm for 10 minutes to obtain plasma. The plasma was decanted into sample bottles and stored in a refrigerator until needed. The liver was immediately removed, washed in ice cold 1.15% KCl solution, and then 50 mg of the wet tissue of liver was weighed accurately and homogenized separately in sample tubes containing 0.25 M sucrose solution. The resulting liver homogenates was centrifuged at 10,000 g for 15 minutes. The supernatants were collected and stored at 4 ºC until required for use.

Analytical chemicals

The liver marker enzymes were carried out using Randox kit products of Randox Laboratories, Ardmore, United Kingdom. All other chemicals were of analytical grade and were supplied by Uche Scientific Research Laboratories, 21, Iga-idunganran Street, Idumota, Lagos State, Nigeria.

Biochemical quantification and analysis

The biochemical assay was carried out in two dimensions, by measuring the liver maker enzymes both in the plasma and liver as well as measuring the antioxidant status in both tissues. The marker enzymes in both plasma and liver tissue homogenate were measured using established methods as described by the following: Creatinine kinase (CK) (Henry, 1979), Aspartate aminotransferase (AST) (Bergmeyer *et al*.,

1985), Alanine aminotransferase (ALT) (Reitman and Frankel, 1957), Alkaline phosphotase (ALP) (Teitz and Shuey, 1986), γ-glutamyl transferase (GGT) (Rosalki and Tarlow, 1974) and Lactate Dehydrogenase (LDH) (Teitz, 1995).

Determination of the antioxidant status in the plasma and liver was performed using published methods. The liver lactoperoxidase (LPO) was estimated by Thiobarbituric acid reacting substance (TBARS) method as described by Varshney and Kale (1990), while reduced glutathione (GSH) was determined by a colorimetric method using Ellman's reagent as described by Boyne and Ellman (1972). The activity of glutathione-S-transferase (GST) was estimated by the method of Habig *et al*. 1974). Superoxide dismutase (SOD) activity was assayed utilizing the technique of Fridovich (1989) while the plasma and tissue Catalase (CAT) were assayed spectrophotometrically at 240nm as described by Aebi (1984) by monitoring the H_2O_2 decomposition.

Histological studies

The method of Raghuramulu *et al*., (1983) was adapted for this examination. A portion of the liver was fixed in formalin, routinely processed and embedded in parafin wax. Sections were cut at 5mm thickness, stained with haematoxylin and eosin and examined by light microscopy.

Statistical Analysis

The mean and standard deviation (Mean± SD) value of all the parameters were determined for each group. Analysis of variance was employed followed Duncan multiple range test for difference between individual treatment group. Difference were considered statistically significant at $p < 0.05$.

RESULTS

Table 1 shows that there was a significant and dose-related decrease ($p < 0.05$) in body weight gain in all Groups treated with $NiCl_2$ when compared with the control. Administration of aqueous extract ($p < 0.05$) showed improved weight gain when compared with the toxicant treated group alone, but this was not statistically significant ($p > 0.05$) when compared with the control. The aqueous extract of *Myristica fragrans* showed no significant lethal effect up to a dose of 2000 mg/kg body weight indicating that LD_{50} should be higher than this dose. The results shown in Table 2 indicates that administration of $NiCl_2$ at 0.75 mg/kg and 1.5 mg/kg concentrations caused a significant ($p < 0.05$) increase in plasma aspartate aminotransferase (AST), Alanine aminotransferase (ALP), Alkaline phosphotase (ALP), γ-glutamyl transferase (GGT), Creatinine kinase (CK) and lactate dehydrogenase (LDH) after 30 days of treatment. However, animals pre-treated with the aqueous extract of *Myristica fragrans* (AEMF) decreased the enzyme concentration to near control level which was significantly ($p < 0.05$) different for AST, ALT, ALP, CK, GGT, and LDH. This effect was also dose-dependent. It was evident from our results (Table 3) that pretreatment with 200

and 300 mg/kg of AEMF significantly delayed the induced hepatic lipid peroxidation when compared with the toxicant groups. The ingestion of $NiCl_2$ alone elicited statistically significant increase in thiobarbituric acid reactive substances level in the liver in a dose related manner. Administration of the toxicant caused 99% and 158% increase respectively, when compared to the control. Observation from Table 3 indicated 14% and 40% reduction upon treatment with 200 mg/kg and 300 mg/kg of AEMF when compared with the $NiCl_2$ treated groups. In addition, the estimation of the non-enzymic (GSH, GST, GPx) and some enzymic (SOD, CAT)

activities revealed a significant decrease ($p<0.05$) upon treatment with $NiCl_2$. However, these were maintained to near control level on administration of 200 mg/kg and 300 mg/kg of AEMF.

Histological examination for the toxicant-treated groups reveals hepatocellular apoptosis associated with a mild lymphocytic infiltrate, congestion of blood vessels with visible lesion. The above conditions were not observed in the extract treated groups except for mild lesion and infiltration of inflammatory cells. Histological examination of extract treated groups, seem not to vary much from control.

Table 1: Changes in body weight of animals upon administration *of aqueous extracts of Myristica fragrans*

Treatment	Initial Weight (g)	Final Weight (g)	Weight Change (%)
Group I	144.56±3.58	155.44±3.00	7.01 ± 2.57
Group II	140.42±1.77	135.96±2.73	-3.28 ± 2.99*
Group III	146.04±1.88	137.02±2.70	-6.58 ± 2.22*
Groups IV	143.54±2.03	141.90±3.36	-1.07 ± 1.95*
Group V	139.20±3.20	143.46±3.08	2.99±3.03*

All values were expressed as mean ± SD for n=6; *significantly ($P<0.05$) decreased compared to control.

Table 2: The effects of *Myristica fragrans* on biomarker of hepatic damage in Plasma

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	CK (IU/L)	GGT (IU/L)	LDH (IU/L)
Group I	52.4±3.40	115.1±6.23	152.1±5.17	28.8±4.97	102.8±2.55	8.3±2.30
Group II	87.3±2.36*	173.5±6.27*	212.2±8.14*	41.2±7.48*	121.3±4.97*	19.0±1.16*
Group III	122.5±3.30*	216.1±11.42*	348.2±15.08*	78.40±5.82*	129.5±4.11*	19.3±2.28*
Group IV	67.6±8.11	119.6±5.44	169.6±10.33	32.6±3.72	103.6±2.17	7.1±3.35
Group V	55.3±5.54	128.3±7.51	160.2±7.15	19.0±1.39	101.0±1.29	7.9±2.21

Values are express as Mean ± SD of 6 animals per group (n=5). Values with * are significantly different ($p<0.05$) when compare with the control.

Table 3: Effect of the aqueous extract of *Myristica fragrans* on liver lipid peroxidation and hepatic antioxidant status.

Treatment	LPO (MDA mM/cm)	GSH (mg/ml)	GPx (mmole/min/ mg protein)	GST (mmole/ min/ mg protein)	SOD (units /min/mg protein)	CAT (mmole /min/ mg protein)
Group I	70.31±4.2	14.83±2.5	102.7±8.3	8.22±1.27	33.27±2.7	1.33±0.15
Group II	139.93±7.3 (99%)[*]	4.29±0.9 (71%)[*]	39.60±5.4 (61%)[*]	2.81± 0.99 (65%)[*]	11.14±4.5 (67%)[*]	0.77±0.73 (42%)[*]
Group III	181.55±9.1 (158%)[*]	4.99± 1.5 (66%)[*]	33.67±3.4 (67%)[*]	2.33±0.87 (72%)[*]	15.11±3.9 (55%)[*]	0.92±1.09 (31%)[*]
Group IV	119.71±4.0 (70%)[*] (14%)[**]	6.06± 1.7 (59%)[*] (41%)[**]	63.51±2.9 (38%)[*] (38%)[**]	4.99±1.31 (39%)[*] (44%)[**]	29.33±3.1 (12%)[*] (62%)[**]	1.22±0.55 (8%)[*] (37%)[**]
Group V	109.38±3.9 (55%)[*] (40%)[**]	8.59±2.00 (42%)[*] (72%)[**]	65.33±4.0 (36%)[*] (49%)[**]	5.03±0.86 (39%)[*] (54%)[**]	31.97±2.4 (4%)[*] (53%)[**]	1.14±0.60 (14%)[*] (19%)[**]

Values are express as Mean ± SD of 6 animals per group (n=5). Values with *% are change with respect control (Group I). Values with **% are change with respect to toxicant groups (Group IV compared with group II and group V compared with group III).

DISCUSSION

This present study suggests a therapeutic strategy for nickel injury by the use of aqueous extracts of *Myristica fragrans* in a dose-related fashion. The increasing interest in studying the involvement of free radicals in carcinogenesis, has led to the use of plant antioxidant treatment to scavenge free radical antagonism on cells as well as improving the health status of humans (Block, 1992; Neff, 1997; Baubles *et al*., 2000). $NiCl_2$ has being classified as carcinogen Class I by inhalation and through drinking water (Das *et al*., 2008). It is pertinent to know that nickel powder, nickel sulphate, nickel chloride, nickel carbonate and nickel nitrate are five priority substances which have been selected by WHO for the risk assessment of nickel (Nestle *et al*., 2002). The result obtained from Table 1 showed a significant p<0.05 decrease in weight in the Groups treated with $NiCl_2$. This observation is an indication that $NiCl_2$ might have caused severe oxidative stress *in vivo*, which has brought about such drastic reduction in weight change when compared with the control. Previous studies have suggested that nickel may bind to DNA-repair enzymes and generate oxygen-free radical to cause protein degradation *in situ*. This irreversible damage to the proteins involved in DNA repairs,

replication, recombination and transcription could be important for the toxic effects of nickel (Lynn *et al*., 1998). Co-treatment of the toxicant with the aqueous extract of *Myristica fragrans* improved the loss in weight in a dose related manner but not to a significant (p>0.05) extent when compared with the control (Table 1).

In the assessment of liver damage, the determination of enzyme marker levels such as ALP, ALT, GGT, AST, CK and LDH is often used. In necrosis or membrane disruption these enzymes are released into the blood stream and can be measured in the serum or plasma as marker to liver damage (Oyagbmi and Odetola, 2010). Results from Table 2 revealed increased level in the activities of AST, ALT, ALP, CK, GGT, and LDH upon treatment with $NiCl_2$. Elevation of plasma enzymes are indicative of cellular leakages and hence loss of functional integrity of cell membrane in the liver (Drotman and Lawhorn, 1978). GGT is one of the enzymes used in monitoring liver damage and the administration of the different doses of toxicant might have resulted in the proliferation of the smooth endoplasmic reticulum, which in turn secreted GGT, causing the marked increase in the

plasma activity. The co-administration of the aqueous extract of *Myristica fragrans* at different doses caused a significant inhibition in the level of ALT, AST, ALP, CK, GGT, and LDH (Table 2).

The assessment of lipid peroxidation (MDA) on $NiCl_2$ treatment showed that the toxicant when compared with the control significantly ($p<0.05$) escalated the level of MDA in a dose dependent manner by 99% and 158%. In rats, the parenteral administration of nickel chloride enhances lipid peroxidation in liver, kidney, and lung, when fresh tissue homogenates was measured by the thiobarbituric acid reaction for malondialdehyde (MDA) (Sunderman *et al.*, 1985). In another study, MDA level was also found to be significantly elevated in serum of nickel chloride-treated rats (Chen, 1998).
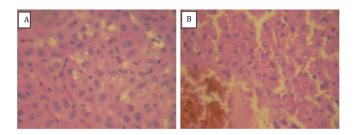

Figure 1: Histopathological damage observed in the liver treated with toxicant. (A): 0.75mg/kg $NiCl_2$: A mild hepatocellular apoptosis associated with a mild lymphocytic infiltrate, (Hematoxylin eosin stain, × 100). (B): 1.5mg/kg $NiCl_2$: The periportal area is surrounded by a mild inflammatory infiltrate composed of lymphocytes and plasma cells (mild periportal hepatitis), congestion of blood vessels with visible lesion (Hematoxylin eosin stain, ×100).
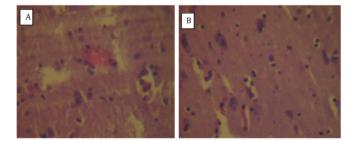

Figure 2: (C): Liver cell treated with 200mg/kg of AEMF, showing slight haemorrhage with no degenerative changes (Hematoxylin eosin stain, ×100). (D): Hepatocytes treated with 300mg/kg of AEMF, showing mild lesion with no degenerative changes (Hematoxylin eosin stain, ×100).

It is evident from this study (Table 3) that pretreatment with AEMF significantly ($p<0.05$) decreased $NiCl_2$ induced liver lipid peroxidation by 14% and 40% respectively when compared with the groups that received only $NiCl_2$, hence exhibiting a dose dependent effect. Also, increasing the concentration of AEMF (from 200 to 300g/kg) non-significantly delayed the lipid oxidation when compared with the control. The concentration of MDA as an index of lipid peroxidation was increased in rats treated with 0.75 and 1.5 mg/kg $NiCl_2$ alone, but pretreatment with aqueous extract of *Myristica fragrans* (AEMF) demonstrated decline in its formation. AEMF might have decreased or suppressed lipid peroxidation though different mechanisms such as radical addition, radical quenching, accepting electrons, electron transfer, radical recombination (Liangli Yu *et al.*, 2002). It is well known that GSH is important in protecting the cell against oxidative stress by hydrogen peroxide reduction, forming conjugates with intermediates or direct quenching of free radicals.

The result from this study (Table 3) demonstrated a significant ($p<0.05$) decrease (by 71% and 66%) in the concentration of GSH in the rats treated with 0.75 and 1.5mg/kg $NiCl_2$ alone when compared to the control. The reduction in the level of GSH promotes the generation of ROS and oxidative stress thereby affecting the structural integrity of the cell (De Level *et al.*, 1996). GSH is largely mediated through the activity of GST, and GPx which also formed abducts with the toxicant. However, the levels of GPx and GST were also significantly ($p<0.05$) reduced in the toxicant treated groups when compared with the control and extract administered groups. Co-administration of toxicant with 200mg/kg and 300mg/kg AEMF as seen in Table 3 indicated significant ($p<0.05$) increased in the level of GSH by 41% and 72% respectively when compared with the toxicant treated groups alone. Our results demonstrate that AEMF co-administration increased the content of GSH in liver tissue and this observation is in agreement with earlier findings of Mohammed *et al.*, 2013 and Chatterjee *et al.*, 2007; and the increased activity of GPx and GST in AEMF-administered group might be correlated to increased availability of its substrate, GSH. A possible reason for the improved increase in the levels of GSH, GST and GPx might be due to the presence of some bioactive substances especially flavonoid, present in the plant. Olaleye *et al.*, 2006 has reported the presence of alkaloids, saponins, anthraquinones, cardiac glycosides, flavonoids and phlobatanins as some of the phytochemical constituent obtained from nutmeg. Furthermore, the activity of enzymatic antioxidants such as SOD and Catalase were studied. The results (Table 3) revealed a dose–dependent decrease in the activities of liver SOD and CAT when treated with $NiCl_2$. The decrease might be as a result of increase H_2O_2 induction in response to the oxidative stress in the hepatocyte. However, hepatoprotective capacity of the co-administration of extract was observed in the significant increase in the activity of liver catalase and superoxide dismutase.

Evidence from the histopathological sections of liver (Figure 1), showed increased tissue damage in $NiCl_2$ alone administered rats, whereas the damage was reduced in groups treated with the aqueous extract of *Myristica fragrans* (AEMF) (Figure 2). Histopatological study of liver from the control group animals showed a normal hepatic architecture with distinct hepatic cells with no degenerative changes. Pretreatment with 200 mg and 300 mg of the extract showed significant changes from toxicant treated group alone with mild degenerative changes from the control. Tissues with scarce apoptotic bodies were observed in the rats that were co-treated with the extracts at different doses. The administration of the AEMF might have culminated into tissue repairs or recovery as observed upon the co-treatment of the extract of *Myristica fragrans* with $NiCl_2$.

Conclusion

The result of this study demonstrated that aqueous extract of *Myristica fragrans* (AEMF) has significant action against $NiCl_2$ induced hepatic-toxicity. The hepatoprotective effect of *Myristica fragrans* might be attributed to its antioxidant principle as well as free radical scavenging properties. Hence, as a preliminary study using low doses, we intend to carry out detailed studies on the mechanism of action as well as plant characterization.

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