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Research Article

GC-MS Analysis of Bioactive Compounds and Safety Assessment of the Ethanol Extract of the Barks of *Holarrhena pubescens* Wall. ex.G.Don (Family Apocynaceae): Sub-Acute Toxicity Studies in Swiss Albino Mice

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ABSTRACT

Background: *Holarrhena pubescens* Wall. ex G. Don belongs to the family Apocynaceae and has several therapeutic applications in traditional medicine. This plant has various pharmacological properties such as antihelmintic, antidiuretic and antidiabetic. One of the major concerns, as they are used, is the lack of adequate pharmacological and toxicological data to support their uses. **Objective:** The present investigation was carried out to evaluate the safety of an ethanolic extract of *Holarrhena pubescens* Wall.ex.G.Don (Apocynaceae) by determining its potential toxicity after oral administration for 28 days. **Methods:** In sub-acute toxicity, the extract at the doses of 250, 500 and 1000mg/kg, bw was administered orally for 28 days. After 28 days of treatment, the mice were decapitated; brain was homogenized for evaluating oxidative stress. The brain was fixed in 10 % formalin and processed for histopathological examinations. Phytochemical analysis of the plant extract was performed by (GC-MS). **Result:** In the sub-acute study in mice, daily oral administration of HP resulted in a significant increase in the lipid peroxidation of treated animals and a decrease in enzymes activity of CAT, SOD, GPX and GR in both, males and females mice. Histopathological analysis showed alterations in the mice brain cortex. From the GC-MS analysis of the plant extract, it was evident that major phytochemicals were present in the ethanol extract of HP. Some major phytochemicals namely, conessimine (17.81 %); lup-20(29)-en-3-one (16.50%); piperidine, 2-(tetrahydro-2-furanyl)-(6.44%); lup-20(29)-ene-3, 28-diol, (3.beta.) (4.82%) and 17-(1, 5-dimethyl-3-phenylsulfanyl-hex-4-enyl (4.37%) were found. **Conclusion:** *H.pubsecne* bark ethanol extract was found to be relatively safe in lower doses although at higher doses it can cause lipid peroxidation and damage to the neuronal cell of the brain and should therefore be used with caution.

Key words: *Holarrhena pubescens,* Sub-acute toxicity, Mice, Oxidative stress, GC-MS.

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INTRODUCTION

Herbal medicines play a major role in health care systems worldwide. The plant-derived medicinal products have been used in modern medicinal products through the use of plant material as indigenous knowledge or as a traditional health care syste [1]. Herbal medicine use increased globally for a public health problem and discover new product and their safety recognized. Some of this medicine are untested these are also not monitored, the lack of knowledge on the possible toxic effect of traditional medicinal plants to the consumers[2]. The health authorities' questions about the discovery and production of medicinal plant drugs; the pharmaceutical and patient industries should be considered[3]. Many medicinal plants considered to have different conventional applications have shown toxicity after acute and sub-acute treatment[4]. on toxicological examination. Therefore, toxicological tests were performed to predict the protection for human use of herbal medicines and other plant-derived products.

Holarrhenapubsescens Wall. ex G.Don (family Apocynaceae), commonly referred to as Tellicherry Bark (English), Kurchi (Hindi), is a small deciduous tree with white flowers. It grows in the tropical Himalayas and can be found all over India[5]. Nearly every part of HP, such as stem, base, bark, and seeds, is known to have different medicinal activities. Crude extracts of HP are stated to have varying pharmacological properties. HP stem bark extracts demonstrate antimicrobial activity[6]. For anti-diabetic activity, anti-hyperglycemic and anti-hyperlipidemic activity, seed ethanol, methanol, aqueous extracts of HP have been studied[7]. An HP plant's seed of hydro-methanol extract reported having an inhibitory activity of alpha-glucosidase in starch charged rats[8]. *In-vitro* studies have anthelmintic activity on the aqueous and ethanol bark extracts from HP[9]. It has been revealed that menthol extract from the HP bark has antimutagenic activity[10]. Seed ethanol extract exhibited anti-hypertensive activity[11]. The anticancer activity has been shown to have ethanol, hydro-ethanol, and aqueous extract of HP leaves[12].

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The large number of phytoconstituents was found in various parts of HP such as conessine, conkurchine, kurchine, pyrrolizidine, holarrhemine, holarrhenine, kurchicine and conkurchinin. Conessine is reported to have sterilant, antifeedant and growth inhibitory effect[13,14].The steroidal alkaloid of HP has also been found to decrease the acetylcholinesterase activity[15]. Another earlier study by Chaturvedi and Singh suggested that the HP bark powder induced numerous side effects such as nausea, nervousness, insomnia, flatulence, constipation, syncope, vertigo, fatigue, xerostomia, anxiety and body lightness[14]. HP bark isolated steroidal alkaloid conessine showed high activity against malaria[16]. A previous toxicity study has been reported that sub-chronic toxicity study at the dose 270 and 540mg/kg caused hepatotoxicity and the plant alkaloid pyrrolizidine have reported toxic to the liver [9,13].

Since no reports on the neurotoxic consequences of long-term intake of HP Bark extracts have been released, further experiments are needed. In certain cases, the current research was conducted to determine the toxic effects of the nervous system based on incomplete information about the consumer's comment about headaches and constipation after ingestion of HP bark. Oral consumption of the ethanol extract of the bark of HP on biochemical, and histopathology.

MATERIALS AND METHODS

Plant extract preparation

The extract preparation of the plant was carried out by the method as described by Veerappan et al.[17] The plant materials were shade dried under room temperature for about 45 days. The shade –dried plants were powdered using soxhlet extractor and after extraction, the sample was filtered with no. 1 whatmann filter paper and then, by allowing filtrate in the rotatory evaporator it was evaporated to dryness. For the initial weight of the dry plant powder, the yield of the extracts (18.76%, W/W) was calculated. The dried extracts were stored at 4ºC until use. The dried plant extract was weighed and suspended in normal saline to give the required concentration before administration to the experimental animals.

GC-MS analysis

GC-MS analysis of the extracts of HP was carried out in Shimadzu GCMS QP-2010 plus system, comprising AOC-20i +s auto –sampler. RTx-5 Sil MS colum was used for the analysis. The operating condition of the colum was maintained as follows:

Oven temperature program from 60ºC to 250 ºC at a rate of 7 ºC/ min with a Hold time of 3 min and from 250 ºC to 280 ºC at a rate of 10 ºC/min with a Hold time of 2 min and the final temperature was maintained for 20 min. the injector temperature was kept at 260 ºC, the volume of the injected sample was 0.3µl; pressure 73.3kPa, purge flow 3.0ml/min, total flow 16.3ml/min, column flow 1.21mL/min, linear velocity 40.1cmsec, split ratio:10.0; ion source temperature 230 ºC, interface line temperature 270 ºC and scan mass range of m/z 40-650. The compound present was identified by comparing their mass spectra with data from WILEY and NIST 14 (National institute of standard and technology, US)[18,19].

For the analysis via GC-MS was done at Advanced Instrumentation Research Facility (AIRF) JNU, Delhi. Of the GC-MS eluted compound were obtained this were characterized based on their molecular formula, structure, retention time, and peak % area[20].

Animal

Male Swiss Albino mice (25±30g) which were obtained from the Pasteur Research Institute, Shillong was used in the present study. The animals were randomly allocated into groups of 10, 12 or 15 in different polypropylene cages and were maintained for acclimatization for one week. The animals were maintained under standard laboratory conditions of temperature (24±2°C) and humidity (60±5%) with free access to proper food and water ad libitum. All the experimental protocol approved by the Animal Ethical Committee of Assam University, Silchar, India (Ref. No.AUS/IAEC/2017/PC/18).

Sub-acute toxicity study

Experimental design

Repeated oral toxicity study for 28 days was determined according to OECD test guideline 407(OECD, 2008). Swiss albino mice that were used in the present study were divided into three experimental groups (n=5 in each group). For this current review, the dose was chosen from the literature based on the effective dose appropriate to antioxidant and antidiabetic action[21].

Group I. Control mice received distilled water,

Group II. Mice fed orally with 250 mg/kg bw of HP bark extract for 28 day

Group III. Mice fed orally with 500 mg/kg bw of HP bark extract for 28 day

Group IV. Mice fed orally with 1000 mg/kg bw of HP bark extract for 28 day

The biochemical and histopathological parameters were done on the 28th day after the animals were sacrificed to study the cerebral cortex and cerebellum region of the brain.

Food and water consumption

Individual food and water consumption was measured, adjusting for spillage, and recorded weekly to coincide with body-weight measurements. Mean food and water consumption were calculated for each sex/dose level during each weekly interval and overall (Days7–28) testing interval. Animals were allowed ad libitum access to food and water throughout the study[22].

Preparation of brain tissue homogenates

After 28 days of treatment, all the mice were anaesthetized and sacrificed by cervical dislocation; brain tissues were excised, washed decapitation, Brain was immediately removed, weighed, minced and homogenized (10%, w/v) separately in ice cold 1.15% KCl-0.01 M sodium, potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 18,000× g for 20 min at 4 ◦C, and the resultant supernatant was used for subsequent biochemical analyses.

Biochemical analysis

Lipid peroxidation assay (LPO)

The level of lipid peroxidation was estimated by the thiobarbituric acid reaction method described by Ohkawa et al.[23] To 0.2ml of the test sample,0.2ml of SDS,1.5ml of acetic acid and 1.5ml of TBA were added. The mixture was made up to 4ml with distilled water and then it is heated in a water bath at 95°C for 60 minutes. After cooling, 1ml of water and 5 ml of n-butanol: pyridine mixture were added and shaken thoroughly. After centrifugation at 4000 rpm for 10 mins, the organic layer was taken and its absorbance was read at 532nm. The level of lipid peroxide (MDA) was expressed as nmoles of MDA released per gram of wet tissue.

Reduced glutathione estimation assay (GSH)

GSH level in the brain is estimated by the method described by Moron et al.[24] 0.5ml (10%) brain homogenate is mixed with 125µl of 25% TCA so that the protein gets precipitated. The test tubes were then

cooled in an ice bath for 5minutes and the cooled mixture was then diluted with0.6ml of TCA. The above mixture then centrifuged for 10 minutes at 1500 rpm and 0.3 ml of the supernatant resulting from the centrifugation of the said mixture is taken for GSH estimation. The volume of the taken supernatant was made up to 1ml with 0.2M phosphate buffer (pH=8) followed by the addition of 2ml of freshly prepared 0.6mM DTNB and then the intensity of yellow formed was measured at 412nm. The GSH level was expressed as mg/gram tissue.

Catalase activity assay (CAT)

The brain catalase activity was estimated by Sinha method [25]. The assay mixture was prepared by the addition of 4ml of H_2O_2 solution (800µmoles) and 5ml of phosphate buffer to 1ml of the brain tissue homogenate.1ml of the above mixture was taken and 2ml of dichromate / acetic acid reagent was added at 60 sec intervals. The H_2O_2 content of the withdrawn mixture is measured by using the standard H_2O_2 curve.

Superoxide dismutase activity assay (SOD)

Superoxide dismutase (SOD) level in the brain was estimated by the Marklund and Marklund method [26]. 100 µl of brain tissue homogenate was mixed with 300 µl of Tris-HCl, 50 µl of EDTA and 50 µl of freshly prepared pyrogallol and then absorbance of the mixture was measured at 420nm.SOD activities are expressed as units/ml. One unit of (Cu-Zn) SOD activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol.

Glutathione reductase activity assay (GR)

GR activity was determined according to the method of Mavis and Stellwagen[27]. The enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The decrease in absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated as nmoles NADPH consumed/mg protein/min.

Glutathione peroxidase activity assay (GPx)

GPx activity was assayed as described by the method of Wendel[28]. 0.1 ml of tissue homogenate was added to the reaction mixture containing 50mM Sodium phosphate buffer with 40mM EDTA (pH 7), 1mM Sodium azide solution, 1mg of β-NADPH, 1mM of DTT with sodium phosphate buffer, 200mM reduced glutathione, 0.042% of H_2O_2, and the absorbance was recorded at 340nm for 5 min.

Acetylcholine esterase activity assay (AChE)

The activity of AChE was determined by the method of Ellman et al [29]. To 100 µl of brain homogenate, 2.87 ml sodium phosphate buffer (Ph7.9) was added followed by the addition of 25µl of DTNB and 5µl of acetylthiocholine iodide. Change in absorbance rate was recorded at 412nm for 10 minutes with the time interval of 1minute.

Total protein estimation

The total protein content in the brain homogenate was estimated by the method of Lowry et al [30]. A standard graph for protein was plotted with BSA and the unknown amount of protein present in the brain homogenates was estimated from this standard graph.

Sample preparation for Microtomy

For histopathological examination, brain tissues were dissected and fixed in 10% phosphate buffered formalin. After the specimens were dehydrated, they were embedded in paraffin. Tissue sections were then cut and processed for hematoxylin and eosin (HE) staining. The sections were examined and photographed under a light microscope.

Sample preparation for transmission electron microscopy

Immediately after dissection of the area of the brain, it was cut into a small cube of 1-2 mm size. During dissection, the brain sample was washed in the fixative. The samples were fixed in Karnovsky's fixative (3% Glutaraldehyde, prepared in 0.1M cacodylate buffer) for 24 hours at 4°C, then the samples were washed with 0.1M cacodylate buffer. The samples in the buffer are then brought to the TEM Lab., SAIF, for the rest of the sample preparation. Temperatures while transporting the samples were maintained around 4°C.

Statistical analysis

All data (n = 5 animals per group) were statistically analyzed using IBM SPSS Statistics 21 software. Statistical comparison of behavioral analysis and the biochemical test was performed using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The data were expressed as Mean ± S.E.M. The value of $p \leq 0.05$ was considered statistically significant.

RESULTS

GC-MS chromatogram analysis of the ethanolic extract of HP (Figure 1) showed 43 peaks which indicating the presence of 43 phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library, the 43 phytocompounds were characterized and identified (Table 1). The mass spectra of all the phytochemicals identified in the plant ethanolic bark extract of HP were presented in Figure 1. Of the 43 compounds identified, the most prevailing compounds were 2-cyclohexen-1-one, 2-hydroxy-3-methyl-6-(1-methylethyl)- (2.57%), d-allose (2.57%), cyclohexanepropanoic acid, 1, 4, 4-trimethyl-2-oxo-, methyle (0.13%), 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol (0.34%), lidocaine (1.14%), dibutyl phthalate (0.93%), n-hexadecanoic acid (2.57%), palmitic acid, tms derivative (0.31%), 9,12-octadecadienoic acid (z,z)- (0.26%), cis-vaccenic acid (1.00%), octadecanoic acid (0.63%), 2-(2-hydroxyethoxy)phenol, di(3-methylbutyl) ether (0.23%), norethindrone (0.30%), 6-undecyl-5,6-dihydro-2h-pyran-2-one (0.25%), 1,7-octadien-3-amine, 3,7-dimethyl- (1.22%), 1,7-octadien-3-amine, 3,7-dimethyl- (2.15%), con-5-enin-3-amine, n-methyl-, (3.beta.)- (3.02%), con-5-enin-3-amine, n-methyl-, (3.beta.)- (1.76%),

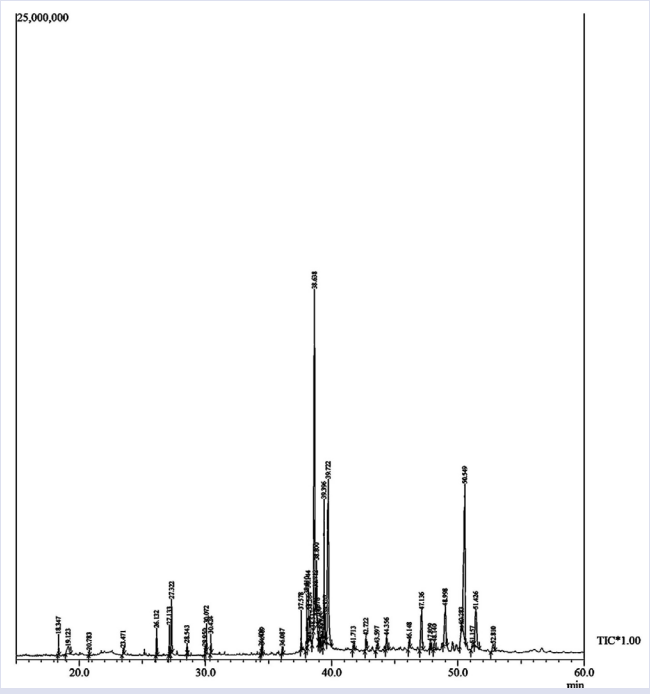

Figure 1: GC-MS analysis of ethanolic bark extracts of HP.

Table 1: GC-MS analysis of an ethanol bark extract of *H.pubescens*.

Peak#	R.Time	Area	Area%	Name of the compound	Chemical formula
1	18.347	1804809	0.67	2-Cyclohexen-1-one, 2-hydroxy-3-methyl-6-(1-methylethyl)-	$C_{10}H_{16}O_2$
2	19.123	3450892	1.28	D-allose	$C_6H_{12}O_6$
3	20.783	341141	0.13	Cyclohexanepropanoic acid, 1,4,4-trimethyl-2-oxo-, methyl e	$C_{13}H_{22}O_3$
4	23.471	926753	0.34	4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$
5	26.132	3066410	1.14	Lidocaine	$C_{14}H_{22}N_{2O}$
6	27.133	2515760	0.93	Dibutyl phthalate	$C_{16}H_{22}O_4$
7	27.322	6918324	2.57	N-hexadecanoic acid	$C_{16}H_{32}O_2$
8	28.543	822008	0.31	Palmitic acid, tms derivative	$C_{19}H_{40}O_2Si$
9	29.950	689005	0.26	9,12-Octadecadienoic acid (z,z)-	$C_{18}H_{32}O_2$
10	30.072	2700268	1.00	Cis-vaccenic acid	$C_{18}H_{34}O_2$
11	30.424	1699500	0.63	Octadecanoic acid	$C_{18}H_{36}O_2$
12	34.409	631141	0.23	2-(2-Hydroxyethoxy)phenol, di(3-methylbutyl) ether	$C_{18}H_{30}O_3$
13	34.489	797536	0.30	Norethindrone	$C_{20}H_{26}O_2$
14	36.087	677540	0.25	6-Undecyl-5,6-dihydro-2h-pyran-2-one	$C_{16}H_{28}O_2$
15	37.578	3281103	1.22	1,7-Octadien-3-amine, 3,7-dimethyl-	$C_{10}H_{19}N$
16	38.010	5787388	2.15	1,7-Octadien-3-amine, 3,7-dimethyl-	$C_{10}H_{19}N$
17	38.144	8123879	3.02	Con-5-enin-3-amine, n-methyl-, (3.beta.)-	$C_{23}H_{38}N_2$
18	38.264	4733879	1.76	Con-5-enin-3-amine, n-methyl-, (3.beta.)-	$C_{23}H_{38}N_2$
19	38.517	758331	0.28	23-Norcona-5,18(22)-dienin-3-amine, n-methyl-,	$C_{22}H_{34}N_2$
20	38.638	47952286	17.81	Conessimine	$C_{23}H_{38}N_2$
21	38.712	924063	0.34	Conanin-3-amine, n,n-dimethyl-, (3.beta.,5.alp	$C_{24}H_{42}N_2$
22	38.800	6394945	2.38	Conessimine	$C_{23}H_{38}N_2$
23	38.876	1077581	0.40	23-Norcona-5,20(22)-dienin-3-amine, n-methyl-,	$C_{22}H_{34}N_2$
24	39.000	1906303	0.71	23-Norcona-5, 18(22)-dienin-3-amine, n-methyl-, (3.beta.)-	$C_{22}H_{34}N_2$
25	39.121	852147	0.32	2-Ethylpiperidine	$C_7H_{15}N$
26	39.245	2360169	0.88	23-Norcon-5-enin-3-amine, n-methyl-, (3.beta.)-	$C_{22}H_{36}N_2$
27	39.396	17345627	6.44	Piperidine, 2-(tetrahydro-2-furanyl)-	$C_9H_{17}NO$
28	39.520	2348258	0.87	Conessimine	$C_{23}H_{38}N_2$
29	39.722	41577934	15.44	Conessimine	$C_{23}H_{38}N_2$
30	41.713	913070	0.34	Octadecanal	$C_{18}H_{36}O$
31	42.722	2370004	0.88	Solanesol	$C_{45}H_{74}O$
32	43.597	1496808	0.56	3.Beta.-dimethylamino-4.beta.-hydroxy-5-pre	$C_{23}H_{39}NO$
33	44.356	3029860	1.13	3,7,12-trihydroxycholan-24-oic acid	$C_{24}H_{40}O_5$
34	46.148	1396922	0.52	Pregnane-3,11,20,21-tetrol, cyclic 20,21-[(1,1-dimethylethyl	$C_{25}H_{43}BO_4$
35	47.136	10664277	3.96	20-Ethynyl-4-pregnene-20-ol-3-one	$C_{23}H_{32}O_2$
36	47.809	1354261	0.50	4,22-Cholestadien-3-one	$C_{27}H_{42}O$
37	48.140	1618342	0.60	<no name>	
38	48.998	11767398	4.37	17-(1,5-Dimethyl-3-phenylsulfanyl-hex-4-eny	$C_{36}H_{54}OS$
39	50.283	1475003	0.55	Lanosta-8, 24-dien-3-ol, (3.beta.)-	$C_{30}H_{50}O$
40	50.549	44412409	16.50	Lup-20(29)-en-3-one	$C_{30}H_{48}O$
41	51.157	1023175	0.38		
42	51.426	12987464	4.82	Lup-20(29)-ene-3, 28-diol, (3.beta.)-	$C_{30}H_{50}O_2$
43	52.810	2229216	0.83	Stigmast-4-en-3-one	$C_{29}H_{48}O$

Table 2: Effect of administration of HP on some biochemical parameters of sub-acute toxicity in albino male mice.

Parameters		Group I (Water)	Group II (250mg/kg)	Group III (500mg/kg)	Group IV (1000mg/kg)
LPO	Cortex	15.16 ± 0.65	15.91 ± 0.41	16.55 ± 0.28	18.01 ± 0.42**
	Cerebellum	14.8 ± 0.58	14.4 ± 0.51	15.4 ± 1.63	21 ± 0.44**
CAT	Cortex	3.54 ± 0.19	3.21 ± 0.17	3.19 ± 0.18	2.81 ± 0.03*
	Cerebellum	4.15 ± 0.39	3.83 ± 0.25	3.6 ± 0.35	2.55 ± 0.22*
SOD	Cortex	4.99 ± 0.36	4.26 ± 0.36	4.15 ± 0.13*	3.48 ± 0.239*
	Cerebellum	5.15 ± 0.39	4.83 ± 0.25	4.6 ± 0.35*	3.71 ± 0.30*
GPx	Cortex	23.93 ± 0.33	23.13 ± 0.62	21.6 ± 0.51*	21.23 ± 0.60*
	Cerebellum	27.4 ± 1.03	25.2 ± 0.58	24.2 ± 0.37*	24.4 ± 0.51*
GR	Cortex	34.52 ± 0.77	33.42 ± 1.30	32.32 ± 0.80	29.78 ± 0.47**
	Cerebellum	35.2 ± 0.86	34.18 ± 0.28	33.37 ± 0.27	32.26 ± 0.57**
GSH	Cortex	26.81 ± 0.60	27.04 ± 0.22	26.04 ± 0.20	25.05 ± 0.62
	Cerebellum	28.4 ± 1.20	27.4 ± 0.51	26.2 ± 0.73	25.2 ± 0.49
AChE	Cortex	3.546 ± 0.17	3.378 ± 0.05	2.80 ± 0.17*	2.51 ± 0.14**
	Cerebellum	3.962 ± 0.26	3.364 ± 0.30	3.04 ± 0.11*	2.58 ± 0.12**

All data are reported as the mean ± S.E.M. for n = 5 per group.ANOVA one way followed by Tukey's Multiple Comparison. *p <0.05: significant differences compared with control 1 (distilled water). CAT (Catalase)= µm H_2O_2 consumed/min/mg protein; SOD (Superoxide dismutase)= U/mg protein; LPO (Lipid peroxidation)= n moles MDA/mg protein; GSH (reduced glutathione)= µm/ g tissue; GR (glutathione reductase) =U/mg protein; GPx (Glutathione peroxidase)= U/mg protein; AChE (Acetylcholine esterase)= U/mg protein.

Table 3: Effect of administration of HP on some biochemical parameters of sub-acute toxicity in albino female mice.

Parameters		Group I (Water)	Group II (250mg/kg)	Group III (500mg/kg)	Group IV (1000mg/kg)
LPO	Cortex	13.08 ± 0.61	13.43 ± 0.22	14.34 ± 0.37	15.21 ± 0.09**
	Cerebellum	10.74 ± 0.29	11.21 ± 0.57	11.94 ± 0.40	13.35 ± 0.25**
CAT	Cortex	2.51 ± 0.24	2.04 ± 0.29	2.09 ± 0.32	1.30 ± 0.12*
	Cerebellum	4.44 ± 0.18	4.18 ± 0.21	3.86 ± 0.19	3.55 ± 0.24*
SOD	Cortex	4.48 ± 0.26	4.41 ± 0.31	3.27 ± 0.18	3.49 ± 0.18*
	Cerebellum	5.18 ± 0.54	4.71 ± 0.20	3.8 ± 0.38	3.44 ± 0.27*
GPx	Cortex	26.86 ± 0.60	26.94 ± 0.25	25.05 ± 0.26*	24.48 ± 0.61*
	Cerebellum	28 ± 0.31	27.82 ± 0.38	26.46 ± 0.31*	26.27 ± 0.33*
GR	Cortex	34.75 ± 0.47	33.29 ± 0.29	33.21 ± 0.34	31.68 ± 0.74**
	Cerebellum	32.91 ± 0.74	30.95 ± 0.60	31.59 ± 0.49	29.61 ± 0.63**
GSH	Cortex	25.81 ± 0.60	24.62 ± 0.46	24.08 ± 1.02	23.82 ± 0.24*
	Cerebellum	25.72 ± 0.33	26.05 ± 0.53	24.63 ± 1.32	24.08 ± 0.50*
AChE	Cortex	3.02 ± 0.12	2.72 ± 0.12	2.24 ± 0.24*	2.03 ± 0.17**
	Cerebellum	3.6 ± 0.09	3 ± 0.09	2.8 ± 0.30*	2.43 ± 0.24**

All data are reported as the mean ± S.E.M. for n = 5 per group.ANOVA one way followed by Tukey's Multiple Comparison. *p <0.05: significant differences compared with control 1 (distilled water). CAT (Catalase)= μm H_2O_2 consumed/min/mg protein; SOD (Superoxide dismutase)= U/mg protein; LPO (Lipid peroxidation)= n moles MDA/mg protein; GSH (reduced glutathione)= μm/ g tissue; GR (glutathione reductase) =U/mg protein; GPx (Glutathione peroxidas)e= U/mg protein; AChE (Acetylcholine esterase)= U/mg protein.

23-norcona-5,18(22)-dienin-3-amine, n-methyl- (0.28%), conessimine (17.81%), conanin-3-amine, n,n-dimethyl-, (3.beta.,5.alp (0.34%), conessimine (2.38%), 23-norcona-5,20(22)-dienin-3-amine, n-methyl- (0.40%), 23-norcona-5,18(22)-dienin-3-amine, n-methyl-, (3.beta.)- (0.71%), 2-ethylpiperidine (0.32%), 23-norcon-5-enin-3-amine, n-methyl-, (3.beta.)- (0.88%), piperidine, 2-(tetrahydro-2-furanyl)- (6.44%), conessimine (0.87%), conessimine (15.44%), octadecanal (0.34%), solanesol (0.88%), 3.beta.-dimethylamino-4.beta.-hydroxy-5-pre (0.56%), 3,7,12-trihydroxycholan-24-oic acid (1.13%), pregnane-3,11,20,21-tetrol, cyclic 20,21-[(1,1-dimethylethyl(0.52%), 20-ethynyl-4-pregnene-20-ol-3-one (3.96%), 4,22-cholestadien-3-one (0.50%), 17-(1,5-dimethyl-3-phenylsulfanyl-hex-4-eny (4.37%), lanosta-8,24-dien-3-ol, (3.beta.)- (0.55%), lup-20(29)-en-3-one (16.50%), lup-20(29)-ene-3, 28-diol, (3.beta.)- (4.82%), stigmast-4-en-3-one (0.83 %). The composition determined for this ethanol extract corresponds to 100% of the entire GC-MS chromatogram.

Overall (Days 7–28) average weekly food consumption for male and female mice at 250, 500 and 1000 mg/kg/ day compared with the control group. A statistically significant decrease was observed in the food consumption on days 21–28 in treated male and female mice in group III (*P<0.05* and *P<0.05*) and IV(*P<0.05* and *P<0.05*), but on Day 7-14, the food intake statistically significant differences were not observed in all treated groups, when compared with control. Water consumption data showed that there were no statistically significant differences among treated groups, compared with the controls all treated group except in group IV as shown in Figure 2.

In the present study, 28 days repeated administration with HP extracts in treated groups at the dose 1000mg/kg bw produced significant increase lipid peroxidation level than the control group in the cerebral cortex (*P<0.01* and *P<0.01*) and cerebellum (*P<0.01* and *P<0.01*) in both the male and female mice (Tables 2 and 3). Here group II and III showed no significant increase in LPO level of the test against the control group.

The mice administered with the HP plant extracts showed decreased Catalase activity compared with the control group in the cerebral cortex (Tables 2 and 3). Here group II showed no significant decrease in Catalase activity of the test against the control group. The group IV showed a significant reduction in catalase activity in both male and female mice compared with control in the cortex (*P <0.05* and *P<0.05*) and cerebellum (*P <0.05* and *P<0.05*). The group III showed a significant reduction in SOD activity in cortex (*P <0.05*) and cerebellum (*P <0.05*) as compared with their respective control only in the female group. The plant extract was given group IV of male and female mice showed a highly significant decrease in SOD activity in both male and female mice compared with control in the cortex (*P <0.05* and *P<0.05*) and cerebellum (*P <0.05* and *P<0.05*). The group III of female mice showed a significant reduction in GPx activity of the test bearing in the cortex (*P <0.05*) and cerebellum (*P <0.05*) against the control group. The group IV showed of both male and female mice a highly significant decrease in GPx activity compared with control in the cortex (*P <0.05* and *P<0.05*) and cerebellum (*P <0.05* and *P<0.05*). The group IV showed a significant reduction in GR activity of mice brain cortex and cerebellum region (*P <0.01* and *P<0.01*) and female mice (*P <0.01* and *P<0.01*) against the control group in the both male and female.

The mice administered with HP extracts showed decreased GSH activity only in female mice compared with the control group in the cerebral cortex and cerebellum (*P<0.05* and *P<0.05*). The group IV showed a significant decrease of GSH activity in cortex (*P <0.05*) and cerebellum (*P<0.05*) compared to control group. Here plant extract treated both groups of male mice showed no significant decrease in GSH activity of the test against the control group.

The mice administered with HP extracts showed decreased AChE activity than the control group in the cerebral cortex (Tables 2 and 3). Here group III and IV showed significant inhibition of AChE activity of cortex (*P <0.05* and *P< 0.01*) and cerebellum *P<0.05* and *P<0.01*) with against the control group in both male and female mice.

Light Microscopic study of H&E stained section of the cerebral cortex of group I (control) and group II mice showed the presence of normal pyramidal cells in the cerebral cortex. The microscopic examination of H&E stained section of the cerebral cortex of group IV mice both sexes (male and female) showed the presence of pericellular halos surrounding the pyramidal cells with darkly stained cell (black arrow) in the cerebral cortex region (Figure 3). Electron microscopic study of the cerebral cortex of group I mice showed the presence of nerve cell containing the regular euchromatic nucleus. group IV mice showed the presence of irregular nucleus containing condensed chromatin Plant extracts treated group IV showing the altered cristae (*) from the cortex of mitochondria with swelling (Figure 4).

DISCUSSIONS

Toxicity studies are considered vital components of herbal medicines' safety and provide evidence before further investigation in clinical

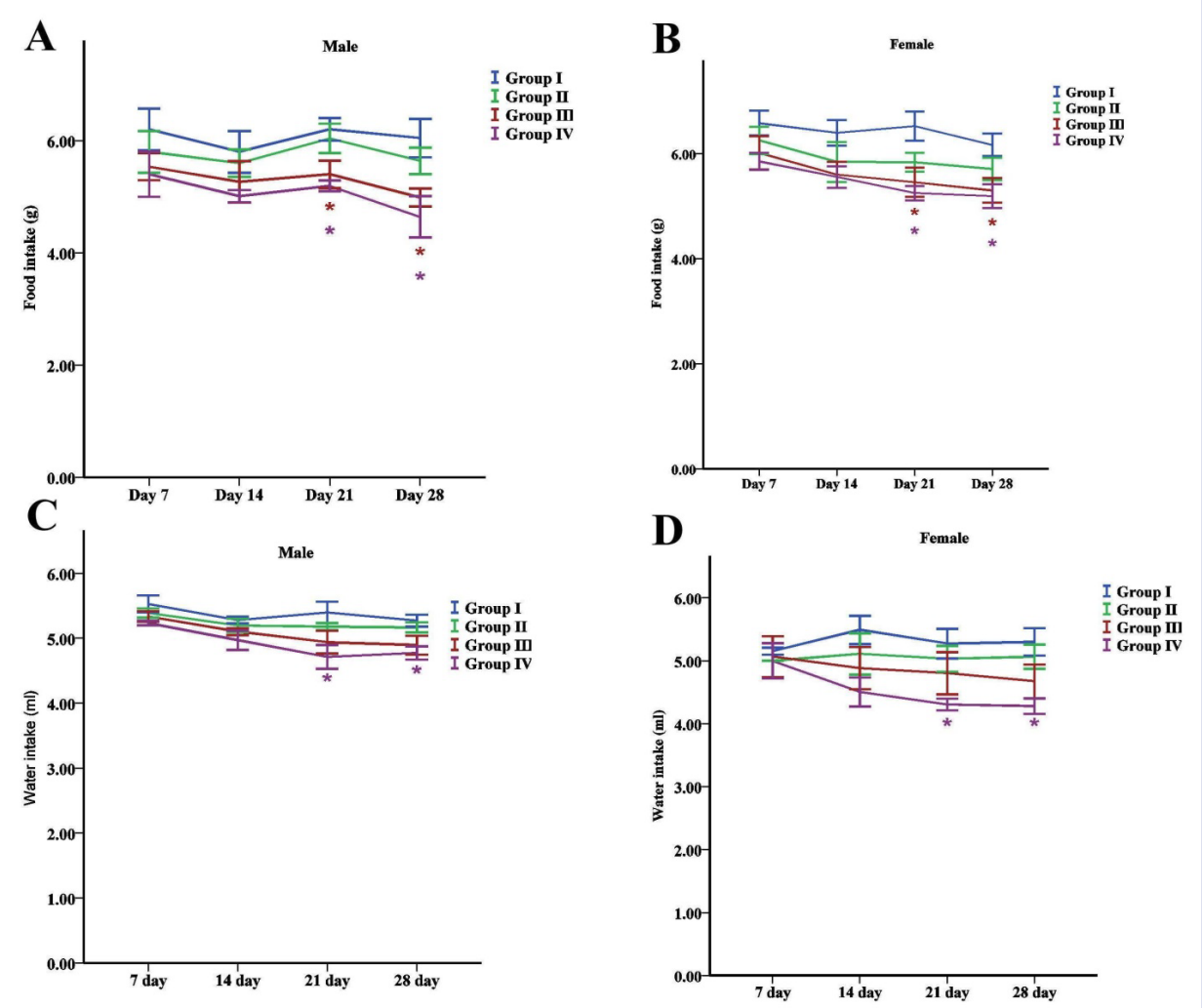

Figure 2. Effect of HP on food and water intake of male and female mice during the 28 days experiment. Data are expressed as mean ± SEM; n = 5. ANOVA one way followed by Tukey's Multiple Comparison. *p <0.05: significant differences compared with the control group.

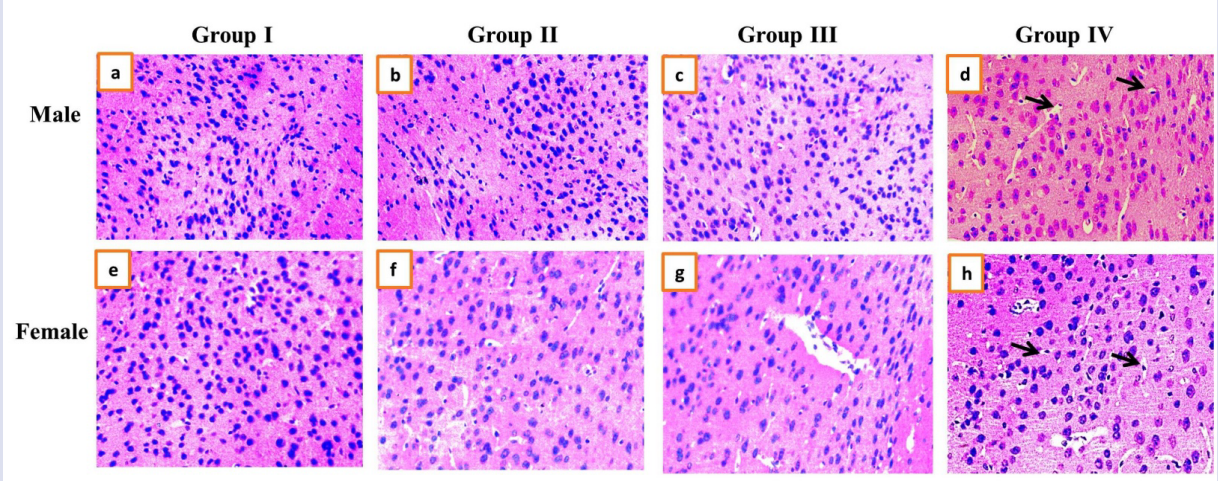

Figure 3: Histological evaluations of cerebral cortex in sub-acute toxicity study of *H. pubescens*. Photomicrograph of a histological section of the cerebral cortex of all treated group mice showing normal histological structure. Photomicrograph of a histological section of the cerebral cortex of group IV showing extracellular vacuoles (↑) in both sex. Scale bars: 100 µm.

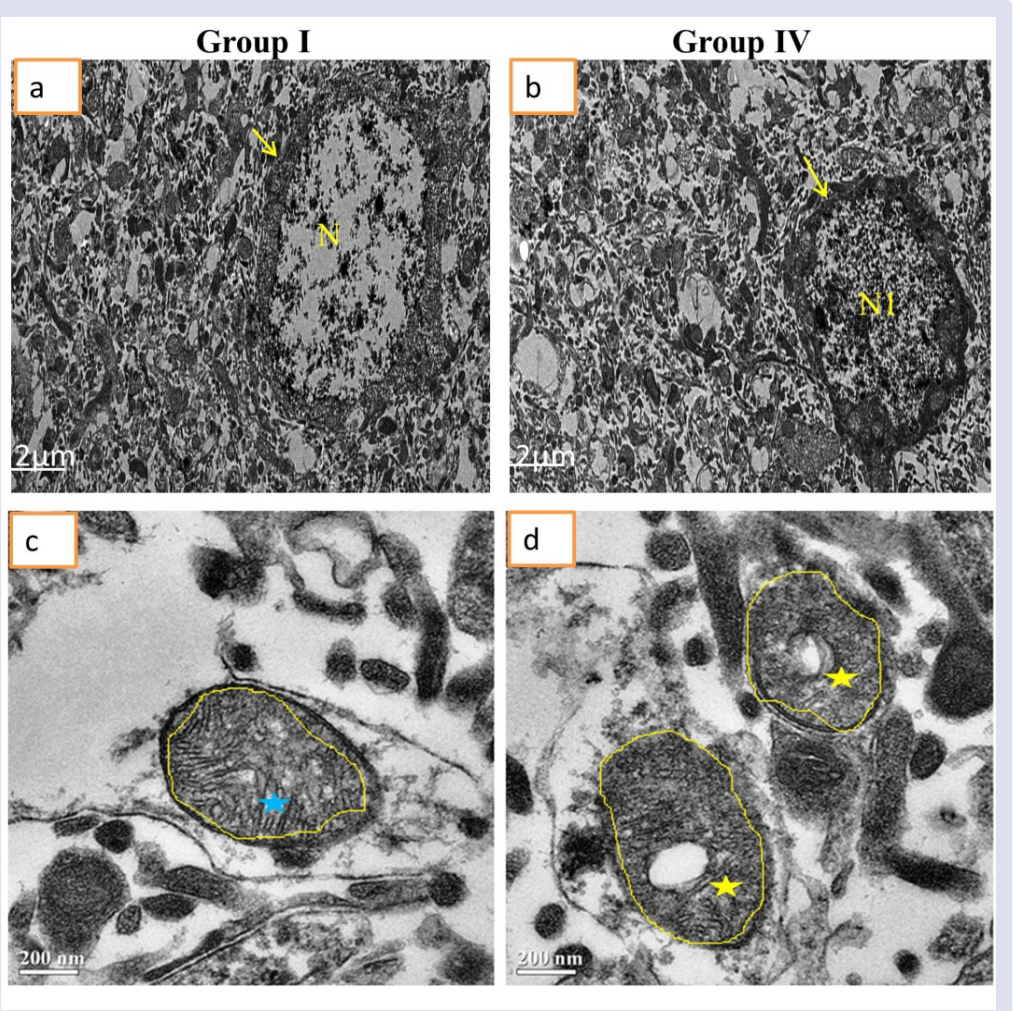

Figure 4: Electron micrograph of a section of the cerebral cortex of group I mice showing a nerve cell containing the regular euchromatic nucleus and normal shape mitochondria. Plant extract-treated group IV showing an irregular nucleus having peripheral condensed chromatin (*) in the brain cortex region. The group IV represents the altered cristae (*) from the cortex of mitochondria with swelling.

trials. Although herbal extracts have been reported to possess multiple bioactivities and potential extensive applications the probable side effects are often neglected[31]. Besides, it is also a useful parameter to investigate the therapeutic index of drugs and xenobiotic[32]. HP is a plant used worldwide in traditional medicine for the treatment of various ailments. Apart from numerous pharmacological beneficial activities of HP, comprehensive knowledge concerning the toxicological effect of the stem bark has not been examined in the nervous system.

The GC-MS analysis of an ethanolic extract of bark of *Holarrhena pubescens* Wall.ex.G.Don revealed the presence of 42 compounds. N-hexadecanoic acid (having peak area value 2.57%) showed anti-oxidant, anti-androgenic, hypocholesterolemic and 5-alpha reductase inhibitory properties. Octadecanoic acid (0.63 %) produced 5-alpha reductase inhibitory and hypocholesterolemic effects[33]. lup-20(29)-ene-3, 28-diol or botulin (4.82%)was known to possess potent anti-proliferative, apoptotic, antimetastatic activities. Triterpenoids, a class of organic compounds which were known to produce antimicrobial and anticancer activities against various cancer types[34]. Lup-20(29)-en-3-one (16.50 %) which was identified in the present study is one of the compounds belonging to triterpenoids. Stigmast-4-en-3-one (0.83 %) was known to possess hypoglycemic property. Cis-vaccenic acid (1.00 %) showed antimicrobial property[35]. On the other hand, among the 42 compounds identified from the ethanolic bark extract of HP, some compounds were known to cause toxic biological effects. For instance, conessimine (17.81 %), a steroidal alkaloid present in the ethanolic bark extract of HP was found to act as a highly potent AChE inhibitor. Conessimine has earlier reported as a component of ethanolic bark extract of *Holarrhena antidysenterica*[36]. Lidocaine (1.14 %) was known to cause morphological neuronal changes in CA3 region of the hippocampus and basolateral amygdala and possibly an inhibition -excitation imbalance. Moreover, disruption of Ca^{2+} homeostasis which results from the interaction of lidocaine with the membrane component can cause neuronal damage[37]. Dibutyl phthalate (0.93 %) was known to induce anxiety, stress, neurobehavioral adverse effects and memory dysfunction in mice. It was also found to cause hippocampal malfunctioning by inducing significant dentate gyrus granular cell abnormalities such as nuclei size reduction and condensation.[38] Piperidine alkaloids (6.44 %) were due to their ability to desensitize nicotinic acetylcholine receptors. Ligand-gated cation channels which mediate the action of acetylcholine in excitatory neurotransmission within the peripheral and central nervous system are targets of nAChRs. Acute toxicity caused by Piperidine alkaloids was known to produce musculoskeletal deformities in neonatal animals[39].

The sub-acute examination confirmed that 28 days of HP administration showed no death or clinical symptoms of toxicity. Determination of the parameters of food and water consumption is critical in the analysis of

the safety of a therapeutic product because the proper intake of nutrients and water is necessary for the physiological status of the animals and Implementation of a correct response to the substance tested rather than a "bad" response due to insufficient dietary conditions[22]. The present result revealed that treated animal-dose dependent plant extract decreases the intake of food and water during the processed period. Accordingly, these findings are considered to be toxicologically significant.

Oxidative stress is an imbalance between the productions of reactive oxygen species (ROS) and antioxidant enzyme. The antioxidant enzyme of the cell's ability to reduce ROS, detoxify reactive intermediates and damage repair that may occur in cellular molecules. This imbalance may occur as a result of increased ROS production, a decrease in defence mechanisms. Major biomarkers of oxidative stress include changes in antioxidant enzyme activity and increase of oxidative damage of cell's[40,41]. The brain is particularly susceptible to lipid peroxidation, Due to the presence of high levels of polyunsaturated fatty acids and iron. Oxidative damage in cells and tissues is mainly manifested as Lipid peroxidation[42]. Lipid peroxidation is indicated by the presence of Malondialdehyde (MDA) in tissues. The measurement of MDA content provides an idea of oxidative injury[43]. Therefore a gradual loss of cell membrane integrity, disruption in membrane transport function and alterations in the cellular ion homeostasis[44]. The present study revealed that with the increase in the dose of administration of HP (1000 mg/kg bw) the MDA level in the brain also increases as compared to the control group (Group I).

SOD is the first enzymatic antioxidant which removes the superoxide anion, by catalyzing the dismutation of superoxide radicals, which generate hydrogen peroxide and O_2. Another antioxidant enzyme, catalase detoxifies H_2O_2 into water and O_2. The excessive accumulation of H_2O_2 can lead to the development of toxic responses in the cellular system[45]. The present study revealed as the doses of HP extract administration increases, catalase activity in the brain of treated mice decreases accordingly. Decreased catalase and SOD activity might be due to depleted GSH level in the brain. This is because; the activity of SOD depends on reduced glutathione (GSH) level[46].

Glutathione peroxidase (GPx) protects the cells from free radicals attack and hence it mitigates oxidative cell damage. GPx removes both hydrogen peroxide and lipid peroxide by converting them into water and alcohol[47]. The current study revealed a decrease in the GPx activity with the increase of doses of HP extract administration. Since glutathione reductase can directly eliminate ROS or reactive oxygen species, so they are regarded as fundamental enzymatic antioxidants. As a result, a decrease in the activity of these enzymes may lead to the accumulation of superoxide radicals and hydrogen peroxide, which in turn cause toxic effects associated with neurodegenerative disorders[48]. The current study also showed reduced activities of GR and GPx enzyme in the brain of a high HP extract-treated group as compared to the control group. GSH plays an extremely potent role in antioxidant defence because it possesses not only the direct radical-scavenging ability but also is an essential component of GPx system, which eliminates different hydroperoxides without producing free radicals[49]. Here in this present study, there is seen a decrease in the GSH activity with the increase of dose.

Antioxidants play a very important role in controlling the rate of lipid peroxidation and eliminating reactive oxygen species[47]. The decrease in natural antioxidative defences results in free-radical-induced oxidative stress associated with the pathogenesis of some clinical diseases [50]. Lipid peroxidation acts as an important indicator of oxidative cell injury[51]. Reduced the antioxidative deficiencies and elevation of lipid peroxidation level observed in the brain of HP treated mice suggest that sub-acute HP extract treatment in high doses can cause oxidative stress in mice brain. The appearance of dark neurons which is seen in this work reflects a certain phase of apoptosis as they displayed markedly condensed cytoplasm and nucleoplasm[52]. Elevated levels of reactive oxygen species can not only cause obliteration of cellular structure and cell membrane but also initiate apoptotic pathway[53]. Thus, the histological alterations observed in the present study might be due to oxidative stress.

The electron microscopic study of the cerebral cortex of HP treated mice revealed the presence of irregular nucleus having condensed chromatin in the nerve cell. The light microscopic study of the cerebral cortex of HP treated mice showed that the pyramidal cells are surrounded by pericellular halos. The irregular outline and loss of the shape in most of the pyramidal cells in this study could be correlated with the cytoskeletal disorganization noticed by the electron microscope. These affected cells revealed major ultrastructure changes in most organelles indicating cell degeneration. The increase of cytosolic calcium and alteration in mitochondrial permeability next to oxidative stress can cause mitochondrial damage. The mitochondrial damage is reflected in its function leading to rapid degeneration of the cells[54]. The mitochondrial and nuclear disorders were considered to be secondary to direct toxicity on neuronal cells that induced disorder of biochemical events[52].

CONCLUSION

In conclusion, According to our report, it causes lipid oxidation and can cause toxicity in the brain by consuming large doses, as the brain has low antioxidant ability. Our findings disclosed the concept of inhibiting antioxidant enzyme activity in high-dose plant extract and it is supported by the histopathological alteration in mice tissue. Consequently, the use of a sufficient amount of *H.pubsecne* barks extracts at low doses for its traditional use should be considered safe.

AUTHORS' CONTRIBUTIONS

All the authors contributed equally.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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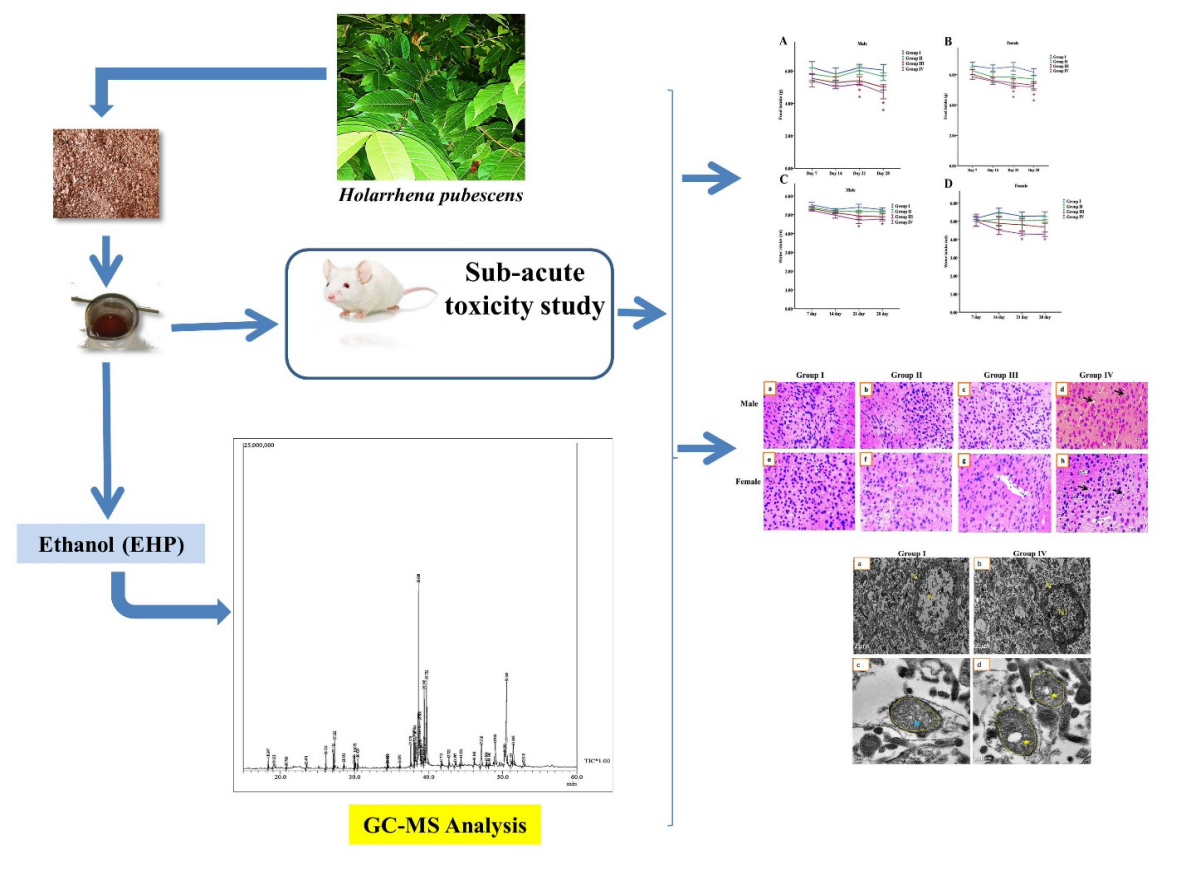
GRAPHICAL ABSTRACT



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