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Sub Acute Toxicity Evaluation of Ethyl Acetate Extracts of Terminalia arjuna (Combretaceae) with Special Reference to Hematological & Biological Parameters

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ABSTRACT

AIM- The aim of the present investigation is to study the Sub acute toxicity study of ethyl acetate extract in male and female rats. MATERIAL & METHODS- The leaves and bark were collected from outfield and also purchased from local markets during the month of July that shows the green color with rough surface. For the extraction, successive solvent extraction methodology was adopted by different solvents as per the polarity index. Subacute oral toxicity studies were conducted by following OECD guidelines- 407 (OECD guidelines, 2001) to evaluate the safety of ethyl acetate extracts. The rats were divided into 7 groups of 10 rats (5 males and 5 females) where group I served as control group and received distilled water only. Groups received ethyl acetate extracts (Low dose-150 mg/kg), Intermediate dose-450 mg/kg, High dose- 1350 mg/kg respectively daily for 28 days. The intermediate dose of most efficacious extract of both the plants were chosen and 1/3rd dose of same was given as low dose and 3 times its dose was given as high dose. It was calculated and given in divided doses in a day. Maximum oral volume fed was not >10 ml/kg. On 28th day after completion of treatment, rats were kept for overnight fasting. Next day, blood was collected from retro-orbital plexus into heparinized and non- heparinized centrifuge tubes for the estimation of haematological and biochemical (glucose, serum creatinine, blood urea nitrogen (BUN), cholesterol, triglyceride (TG), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) parameters respectively. RESULTS- The study on selected plant material, shows, that the difference of two consecutive weighing after drying for 30min. and cooling for 30min. in a desiccators- 0.09 & 0.23 gm for leaves and bark. The Experimental results on selected plant material shows – volume occupied by 1 gm of plant material= 1.62, 1.45 ml for leaves and bark. Any unusual behavioral changes, disturbance in locomotors activity, any untoward clinical signs, sign of intoxication and mortality were not observed at the dose levels tested in sub-acute toxicity test. Moreover, the nature of stool, urine and eye color of all the animals did not changed. Other adverse clinical manifestations were not seen in the experimental animals during the dosing period. CONCLUSION- The sub acute toxicity study did not show any treatment related mortality and adverse effects at 150, 450 and 1350mg/kg p.o. dose administration of extract.

KEYWORDS: Sub acute, Toxicity Evaluation, Ethyl Acetate Extracts, Terminalia arjuna (Combretaceae), Hematological, Biological Parameters.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal (GI) tract, characterized into two main types: Ulcerative colitis (UC) and Crohn's disease (CD). German surgeon Wilhelm Fabry first identified Crohn's disease in 1623, However it was later named after the US physician Burril B Crohn. Ulcerative colitis was discovered in 1859 by the British Physician Sir Samuel Wilks [1]. IBD was traditionally considered as a disease of developed nations, however in recent years a change in epidemiology has been noted. High income countries with greater disease burden and prevalence rates are indicating stabilizing occurrence, simultaneously in the newly developing countries of South America, Eastern Europe, Asia and Africa a rapid rise in cases is noted. These rapid changes occurring worldwide suggests call for global estimates to provide insight into the burden and trends. Moreover, defining the varying incidence and diagnosis of IBD in different geographical regions might provide evidence to the cause of the disease which is not yet clearly known [2]. UC is a gastrointestinal disease that is confined to the colon, where inflammation is involved either in the entire colon or a part of it. However, CD can affect any part of the GI tract from mouth to the anus. Although CD is more severe than UC, the global prevalence is much lower. Approximately 1.86 billion UC patients have been diagnosed globally, with 1.54 billion patients currently receiving treatment and 1.3 million CD patients diagnosed and 0.8 million receiving treatment. Traditional therapies have yielded $4.18 billion for UC and $3.17 billion for CD in annual sales worldwide, and expected to increase further to $6.85 billion and $4.20 billion respectively by 2022 with the approval of various pipeline drugs [3]. The pathological process of IBD is still not clear however it is known to involve a

complex interrelationship between genetic or environmental factors, gut microflora and mucosal immune response [4]. The immune system imbalance and/or altered microbial interactions leads to progression of chronic intestinal inflammation in genetically susceptible hosts when certain environmental factors are triggered. Th1 cells were considered to play an important role in CD pathogenesis and Th2 cells were shown to be involved in pathogenesis of UC. However, newer developments suggest Th17 cell activation and imbalance of Th17/regulatory T (Treg) cells to be a prime component in the development of gut inflammation [5]. The aim of the present investigation is to study the Sub acute toxicity study of ethyl acetate extract in male and female rats.

MATERIALS AND METHODS

Identification and collection of plant material

The leaves and bark were collected from outfield and also purchased from local markets during the month of July that shows the green color with rough surface. The plant leaves were washed thoroughly in tap water, dried in shade, finely powdered and used for successive extraction methods. Plant was identified by the Botanist and herbarium specimen was submitted in Department of Pharmacognosy.

Successive extraction methods:

For the extraction, successive solvent extraction methodology was adopted by different solvents as per the polarity index. The % Yield of the Petroleum ether, Chloroform, Ethyl acetate, Ethanol, Methanol, & Aqueous extract was calculated by using the following formula [6].

$$\% \text{ Yield} = \frac{\text{Net weight of powder in gram after extraction}}{\text{Total weight of leaf powder in gram taken for extraction}} \times 100$$

Phytochemical Screening

Each extract was subjected to qualitative chemical tests to find out the presence or absence of phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins and flavonoids [6-8].

Animals:

Healthy Male wistar albino rats (250-300gm, 10-11 weeks age) were housed in cages with free access to standard rat chow (diet) and water *ad libitum* and acclimatized to the surroundings for one week prior to the experiment. Animals were maintained on a light/dark cycle (12/12hr) at a constant temperature (22º±1ºC) and humidity (55±1). The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Sub acute Toxicity Studies

Grouping and drug administration:

Subacute oral toxicity studies were conducted by following OECD guidelines- 407 (OECD guidelines, 2001) to evaluate the safety of ethyl acetate extracts. The rats were divided into 7 groups of 10 rats (5 males and 5 females) where group I served as control group and received distilled water only. Groups received ethyl acetate extracts (Low dose-150 mg/kg), Intermediate dose-450 mg/kg, High dose- 1350 mg/kg respectively daily for 28 days. The intermediate dose of most efficacious extract of both the plants were chosen and 1/3rd dose of same was given as low dose and 3 times its dose was given as high dose. It was calculated and given in divided doses in a day. Maximum oral volume fed was not >10 ml/kg.

Mortality, clinical signs and assessment of motor and sensory activity:

In subacute toxicity studies, animals were observed with intervals for the first 4 h, afterwards every 6 h for the next 24 h. Second day onwards animals were observed once a daily for any changes in general behavior and physiological activities up to 28 days. During the four-week dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing. The number of survivors was noted. Changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, and unusual respiratory pattern) were observed. Also changes in gait, postureand response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour were recorded. In the fourth exposure week sensory reactivity to stimuli of different types (IPCS, Environmental Health Criteria, 1986) (e.g. auditory, visual and proprioceptive stimuli) [9], assessment of grip strength [10] and motor activity assessment were also conducted. Attention was also directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Hematological and Biochemical parameters:

On 28th day after completion of treatment, rats were kept for overnight fasting. Next day, blood was collected from retro-orbital plexus into heparinized and non- heparinized centrifuge tubes for the estimation of haematological and biochemical (glucose, serum creatinine, blood urea nitrogen (BUN), cholesterol, triglyceride (TG), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) parameters respectively. For Complete Blood Count, non-heparinized blood samples were run on fully automated hematology analyzer PCE 210. Serum glucose was estimated by GOD-POD method and

all biochemical parameters were estimated using Biochemistry analyzer.

Statistical Analysis:

Results were expressed as mean ± standard error of the mean (SEM). Data was analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. For toxicity studies, all the parameters were analyzed statistically using ANOVA, followed by Tukey's Multiple Comparison Test post hoc test. All the tests were done using Graph Pad Prism 6.

RESULTS

Table No. 1: Physico-chemical parameters

S. No.	Determination	Plant Name	
		Terminelia arjuna	
		Leaves	Bark
1	Total ash	8.0±0.14	8.6±0.11
2	Acid insoluble ash	3.12±0.04	2.17±0.17
3	Water soluble ash	4.96±0.11	3.89±0.22
4	Alcohol soluble extract value	5.40±0.13	4.45±0.34
5	Water soluble extract value	3.90±0.16	3.40±0.22
6	Loss on Drying	6.98±0.32	5.52±0.56

Determination of Moisture content

The study on selected plant material, shows, that the difference of two consecutive weighing after drying for 30min. and cooling for 30min. in a desiccators- 0.09 & 0.23 gm for leaves and bark.

Determination of Swelling Index

The Experimental results on selected plant material shows – volume occupied by 1 gm of plant material= 1.62, 1.45 ml for leaves and bark.

% Yield Determination and Characteristic views of extracts

Table 2: %Yield (w/w) of extracts

S No.	Solvent	*Terminelia arjuna*	
		Leaves	Bark
1	Petroleum ether	5.29	3.12
2	Chloroform	5.18	4.15
3	Ethyl Acetate	5.14	6.34
4	Ethanol	11.28	9.56
5	Methanol	5.28	8.56
6	Aqueous	9.80	4.67

Phytochemical Screening

Phytochemical screening of different extracts showed the presence of different phytochemical.

Table 3: Preliminary Phytochemical test for different extracts

S.No.	Test	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Methanol	Aqueous
1.	Carbohydrate Molish test	-	-	-	-	+	+
2.	Glycosides Bronteger test			+		+	+
3.	Alkaloid Mayer test Hager test	-	+ +	+ +	-	+ +	-
4.	Phytosterol +Triterpinoids	+	+	+	-	-	-
5.	Protein + Amino acid Biuret test Ninhydrin test	- -	- -	- -	- -	- -	- -
6.	Phenolic test Ferric test Lead acetate test	-	+ +	+ +	+ +	+ +	-
7.	Flavonoids Alkaline test	-	-	+	+	+	+
8.	Saponin Foam test	-	-	-	-	+	+
9	Mucilage Iodine test Ethanol test						+ +

Note: (+) *ve* indicates positive result, whereas (–) *ve* indicates negative result

Sub acute Toxicity Studies

Effect of extracts on clinical signs and behavioral changes:

The animals fed with the extracts were healthy. Any unusual behavioral changes, disturbance in locomotors activity, any untoward clinical signs, sign of intoxication and mortality were not observed at the dose levels tested in sub-acute toxicity test. Moreover, the nature of stool, urine and eye color of all the animals did not changed. Other adverse clinical manifestations were not seen in the experimental animals during the dosing period. As far as the clinical examinations are concerned, any indices of toxicity were not observed, when compared to the control group. Changes in skin, fur, eyes, mucus membrane and autonomic activity were not observed. Gait, posture, response to handling was all normal during the 28 day period of study. Stereotype behaviour like excessive grooming, repetitive circling, bizarre behaviour like walking backwards were not observed suggesting no change in CNS function. Sensory reactivity to stimuli of different type's auditory, visual proprioceptive stimuli motor activity was unaltered.

Table 4: Hematological parameters of ethyl acetate extract in sub acute toxicity

Hematological parameters	**MALE**				**FEMALE**			
(Normal Values)	**Control**	**Ethyl acetate (mg/kg)**			**Control**	**Ethyl acetate (mg/kg)**		
		150	**450**	**1350**		**150**	**450**	**1350**
RBC(x10^6/mm^3) **(7-10)**	7.42± 1.47	7.866 ± 0.33	7.552 ± 1.23	7.232 ± 3.55	7.712± 1.27	7.32± 1.27	6.96± 1.76	7.13± 1.57
Hb (g/dl) **(11-18)**	12.9± 1.77	13.86 ± 1.56	14.56 ± 1.21	14.23 ± 1.11	14.98± 2.97	13.02 ± 1.62	13.22 ± 1.54	12.85 ± 1.93
MCV(fl) **(36-58)**	49.76± 1.89	49.46 ± 2.67	50.62 ± 1.45	51.01 ± 3.87	50.84± 1.39	48.18 ± 2.21	41.96 ± 9.86	43.59 ± 3.16
MCH(pg) **(17.1-20.4)**	17.24± 2.55	17.6± 1.23	17.9± 1.47	17.5± 3.54	17.7± 2.317	17.48 ± 1.29	18.24 ± 1.44	18.69 ± 1.95
MCHC (g/dl) **(32.9-37.5)**	34.9± 1.98	35.54 ± 2.76	35.44 ± 2.11	36.42 ± 4.22	35.9± 3.57	34.64 ± 2.63	34.94 ± 1.95	35.27 ± 1.83
WBC(x10^3/mm^3) **(6-17)**	9.84± 1.45	11.06 ± 1.89	10.86 ± 2.96	11.94 ± 1.89	8.5± 1.55	9.62± 1.78	8.64± 0.71	8.96± 1.46
Lymphocytes (%) **(65-85)**	65.06± 3.22	73.64 ± 2.34	68.96 ± 1.78	68.58 ± 2.66	69.87± 3.34	71.78 ± 2.55	69.02 ± 2.31	70.39 ± 1.85
Monocytes(%) **(0-5)**	5.02± 2.34	2.78± 1.11	4.56± 1.33	3.48± 2.65	4.82± 2.94	2.98± 0.84	4.64± 1.51	3.79± 0.72

Granulocytes (%) **(9-34)**	19.42± 4.72	13.58 ± 1.87	16.48 ± 1.11	14.63 ± 1.33	13.22± 1.25	14.36 ± 1.83	15.94 ± 2.733	16.01 ± 1.672
PLT(x10^3/mm^3) (500-1300)	573.6± 3.45	634.2 ± 2.34	654.2 ± 3.98	657.1 ± 2.67	581.6± 4.89	659.6 ± 4.66	674.4 ± 6.13	668.3 ± 2.69

Table 5: Biochemical parameters of ethyl acetate extract in sub acute toxicity

Biochemical parameters	**MALE**				**FEMALE**			
(Normal values)	**Control**	**Ethyl acetate (mg/kg)**			**Control**	**Ethyl acetate (mg/kg)**		
		150	**450**	**1350**		**150**	**450**	**1350**
RBS(mg%) (50-135)	130.5± 4.5	109.6± 4.59	117.5± 3.2	121.8± 3.7	130.9± 3.02	114± 3.91	130.5± 3.03	127.5± 2.04
BUN(mg%) (15-21)	18.59± 1.46	18.76± 2.53	20.59± 1.79	19.89± 2.62	19.01± 1.42	19.87± 1.76	19.56± 2.42	21.01± 1.63
Creatinine (mg%)(0.2- 0.8)	0.764± 0.04	0.822± 0.035	0.798± 0.025	0.824± 0.04	0.796± 0.05	0.754± 0.05	0.682± 0.05	0.723± 0.09
Cholesterol (mg%)(40- 130)	87.09± 2.89	89.89± 1.37	95.04± 2.81	93.47± 2.91	92.74± 2.64	89.08± 2.31	94.02± 3.94	91.04± 3.28
Triglycerides (mg%)(20- 114)	69.56± 2.92	84.5± 3.86	81.13± 4.59	82.36± 4.02	78.14± 3.43	85.36± 4.68	91.16± 4.28	88.21± 3.75
SGPT(IU/L) (18-45)	42.7± 3.41	41.16± 2.52	43.37± 1.43	42.28± 1.26	36.82± 2.26	39.22± 2.66	42.45± 2.39	43.16± 2.46
SGOT (IU/L) (74-143)	62.49± 4.73	53.35± 1.29	67.89± 5.72	62.37± 3.42	62.08± 3.85	51.08± 2.85	60.52± 1.22	55.28± 2.92

Data is represented as mean ± SEM. Statistical analysis was carried out using one- way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test.P values <0.05 were considered significant. Normal values are as per CPCSEA guidelines.

DISCUSSION

Inflammatory bowel disease is a chronic relapsing inflammatory disorder involving the gastrointestinal tract. In IBD, the bowel i.e intestine becomes inflamed, leading to abdominal cramps and diarrhea. Incidences have been gradually increasing in India due to adaptation to western living style. Most of the poly herbal formulations face problems in standardization and quality control. Also, it is very difficult to say exactly which plant is responsible for the efficacy or which plant is responsible for the side effect seen. If any clinically proven mono herbal treatment is available, with evidences for its action on the sites involved in the pathogenesis of the disease, coupled with elucidation of the exact mechanism of action responsible for therapeutic action in preclinical studies, it would be a relatively affordable alternative treatment with more acceptability for the patients. The medicinal plants chosen for the study were selected on the basis of the active chemical constituents present and activities reported. Phytoconstituents like natural steroids, phenolic compounds [11], flavonoids [12], saponins [13], terpenoids and alkaloids [14] are well known for their antioxidant and antiinflammatory activities. Preliminary phytochemical screening of the extracts revealed the presence of various phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins and flavanoids in both the medicinal plants.

Currently, toxicology encompasses mainly activities to determine the potential for adverse effects from chemicals, with the objective of assessing hazard and risk to humans and animals. After preliminary positive results of efficacy studies, in order to establish the safety of a new drug, *in vivo* toxicological studies are very essential experiment carried out in animals. We all believe that natural therapy using herbs is safe. But it is also very important to assess scientifically the potential beneficial or adverse effects of all medicinal plants extensively used by us. In order to establish the safety of a new drug, various guidelines are established for preclinical studies. The toxic effects are seen for single dose as well as repeated dose so that their acute effects and chronic effects can be established especially if they are to be used in varying doses for a longer period of time. Hence acute and sub-acute toxicity studies are always invaluable in evaluating the safety profile of phytomedicines.

The sub acute toxicity study allows the establishment of the existence or not of adverse effects, and later for the identification and characterization of the affected organs. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to potentially toxic substances. In addition, the diet if well accepted does not cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. It should not adversely interfere with the nutritional benefits Sub acute toxicity study in our studies revealed that extracts of both the plants at various doses did not appear to retard growth or affect food consumption and utilization. The haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status for both animals and humans. The levels of SGOT and SGPT are good indicators of liver functions. Nephrotoxicity of the drug can be estimated by renal function markers like creatinine and BUN. There were no treatment related changes in the haematological indices. No significant changes were observed due to treatment which indicates that the drug did not interfere with the renal and hepatic functions. The treatment did not show any significant changes in lipid parameters and glucose levels.

CONCLUSION

The sub acute toxicity study did not show any treatment related mortality and adverse effects at 150, 450 and 1350mg/kg p.o. dose administration of extract. Presence of various phytoconstituents viz. steroidal alkaloids, flavonoids, presence of steroidal sesquiterpene hydrocarbons, monoterpene, aliphatic alcohols and triterpenes.

Ethyl acetate extract contain phytoconstituents like alkaloids, flavonoids and terpenes, which are the key factors in the medicinal value of these plants. Thus, the possible mechanism of protective effect might be attributed to these active principles exhibiting anti-oxidant and anti-inflammatory via down regulation of proinflammatory cytokines.

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