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RESEARCH ARTICLE

SAFETY ASSESSMENT OF PARTIALLY PURIFIED HDL ENHANCING PHYTOCOMPONENTS FROM *DESMODIUM GYRANS* DC.

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Key words:-
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Abstract

Desmodium gyrans (Fabaceae) is a less explored plant belonging to medicinally important genera Desmodium. A recent investigation has reported hypolipidemic and HDL enhancing efficacy of crude alcoholic extract (DGM) of the whole plant. Chromatographically purified aqueous fraction (DGMAF) rich in terpenoids and phenolics exhibited HDL enhancing and reverse cholesterol transport (RCT) promoting activity that may impart atheroprotective potential to this active fraction. Present study was aimed to evaluate the acute and sub-acute oral toxicity of DGMAF in Wistar rats of either sex following OECD guide lines to ensure its safety upon long term exposure. Acute oral dose of 5g/kg body weight did not produce any behavioural abnormality, weight changes or mortality over 14 day period. Necropsy studies revealed no incongruity to internal organs. Further, oral sub-acute doses of 100, 250, and 500 mg/kg body wt for 60 days did not show reduction in body and organ weight, food and water intake. Hematological parameters and biochemical analytes inclusive of hemoglobin, urea, creatinine, total and direct bilirubin, serum GOT, GPT, and ALP did not show statistically significant differences between the untreated control and treated groups. No morphological changes were observed in the histological analysis of the major vital organs including liver, kidney, spleen and intestine. The result of this study thus suggests that DGMAF might be non-toxic at normal therapeutic doses.

Introduction:-

Herbal products are attaining popularity as alternative medicine for treatment of various diseases because of its effectiveness, low cost and safety on long term use. The methanolic extract of *D. gyrans* (DGM) has been previously shown to reduce high fat diet induced hyperlipidemia in rats and rabbits with significant increase in HDL fraction (Vipin *et al*., 2015). Further to confirm the RCT promoting efficacy DGMAF has to be tested *in-vivo* using rat models. In previous reports, there is no record in the literature on the toxicity profile of *Desmodium gyrans*. The acute and sub-acute toxicity data are mandatory to predict the safety and effects of long term exposure. The present study was therefore undertaken to determine the toxicity profile of DGMAF on Wistar rats. This study evaluates DGMAF for its toxic effects using hematology, serum parameters, and histopathological changes as toxicity indices.

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Materials and methods:-

Plant collection, authentication and preparation of extract of DGM:-

Plants were collected from Vazhani Forest Range (Thrissur District) and authenticated by Dr. C. N. Sunil (Associate Professor, Department of Botany, SNM College Maliankara, Moothakunnam, Ernakulam). A voucher specimen (SNMH-7012) was deposited in the herbarium of SNM College, Maliankara, Moothakunnam, Ernakulam. Collected aerial parts of plant were cleaned, dried under shade and powdered. The crude aqueous alcohol extract was prepared using 70% methanol in soxhlet apparatus. The extract was filtered and dried by evaporation. The solvent free DGM (1g) was loaded on to silica gel (60 x120 mesh) column (120 x 30 mm) based on polarity. The fraction eluted with distilled water was collected and evaporated to dryness and the solvent free residue (DGMAF) was used for the present study. This fraction (DGMAF) was dissolved in distilled water to a known volume. Animals were administered 5 g/kg b.wt for acute toxicity study, 100, 250 and 500 mg/kg b.wt for sub-acute toxicity study through oral route.

Animals:-

Male and female Wistar rats weighing around 170 to 200 g were purchased from the Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala. They were maintained under standardized environmental conditions (22-28^{o}C, 60-70 % relative humidity, 12 hr dark/light cycle) and fed with standard rat feed (SaiDurga Feeds, Bangalore, India) and water *ad libitum.* All the animal experiments were carried out with the prior permission from Institutional Animal Ethics Committee (ACRC/IAEC/16-06-12) and conducted strictly according to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Acute toxicity analysis of DGMAF:-

The oral acute toxicity of DGMAF was evaluated according to Organization for Economic Co-operation and Development (OECD) guideline. All rats were kept at room temperature with persistent humidity. They were permitted to acclimatize to laboratory conditions for a week prior to the experiment. Drinking water and food were provided *ad libitum* throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 12 hr prior to treatment. Animals were observed for 7 days before oral drug administration. Following the fasting period, body weights of the rats were determined and the dose was calculated in reference to the body weight. After 7 days, a single high dose of 5 g/kg. b.wt of DGMAF (dissolved in distilled water) was administered to three female and three male rats (Average weight: 189 g) by the oral route. Foods were provided to the rats approximately an hour after treatment. The rats were observed in detail for any indications of toxicity effect within the first six hours after the treatment period, and daily further for a period of 14 days. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period. At the end of experimental period the animals were euthanized and necropsy was carried out.

Sub-acute toxicity:-

Wistar rats of both sexes (males and females) were used for the study. During the experimental period of 60 days, the rats were grouped into four groups comprising untreated normal and drug groups such as doses of 100 mg/kg b.wt, 250 mg/kg b.wt and 500 mg/kg b.wt of DGMAF. During the experimental period, food and water consumption and increase in body weight was recorded once weekly. At the end of the experimental period, the animals were fasted overnight and sacrificed on the following day. Blood was collected through cardiac puncture and used for the assessment of hematological parameters and organs including liver, kidney, lung, spleen, heart and brain were excised to calculate relative organ weight as well as histological analysis. Serum was obtained after centrifugation of blood at 1500 rpm for 10 min and used for biochemical analysis.

Hematological parameters such as total count and differential leukocyte count were assessed using whole blood. Hemoglobin was determined using Drabkin's method (Drabkin DL, 1932). Activities of serum marker enzymes such as total and direct bilirubin, glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) were estimated using commercially available kits obtained from Span Diagnostics Ltd (Surat, India). Kidney function tests such as serum creatinine and urea were also measured using kits from Span Diagnostics.

Histological analysis:-
A portion of liver, kidney, spleen and intestine from each group were cut, washed in PBS, fixed in10% neutral buffered formaldehyde solution and then embedded in paraffin wax. Sections round 5-6 microns in thickness were taken and stained using double staining method using hematoxylin (nuclear stain) and eosin (cytoplasmic stain). Slides were then photographed at 400 X magnification.

Statistical analysis:-
Data are presented as mean ± SD of 6 animals. Statistical comparisons are made using a one-way analysis of variance (ANOVA) followed by Dunnett't' test using Graph Pad Instat 3 software (Graph Pad Software, Inc. La Jolla, USA).

Results:-

Acute- toxicity study:-
Single dose of DGMAF (5 g/kg b.wt) was given for acute toxicity based on OECD guideline 420 (Adopted December 2001), Para 19. There was no significant difference between the test groups administered with DGMAF in relation to the normal untreated group. In the acute toxicity analysis, the DGMAF was found to be non-toxic to Wistar rats of both sexes as no mortality or abnormal changes such as changes in skin and fur, eyes and mucus membranes, behavior pattern, tremors, salivation, sleep, coma, moribund, ill health or any visible reaction to treatment were observed during the period of two weeks. The body weight was maintained without much variation (Figure 1) and no diarrhea or hair loss was observed. Also no significant changes were seen in hematological data such as hemoglobin concentration and total count of leukocytes (Table 1). Since there was no toxicity observed, doses above 5gm/kg b.wt is not considered for further assessment and has been assumed as its $LD_{50.}$

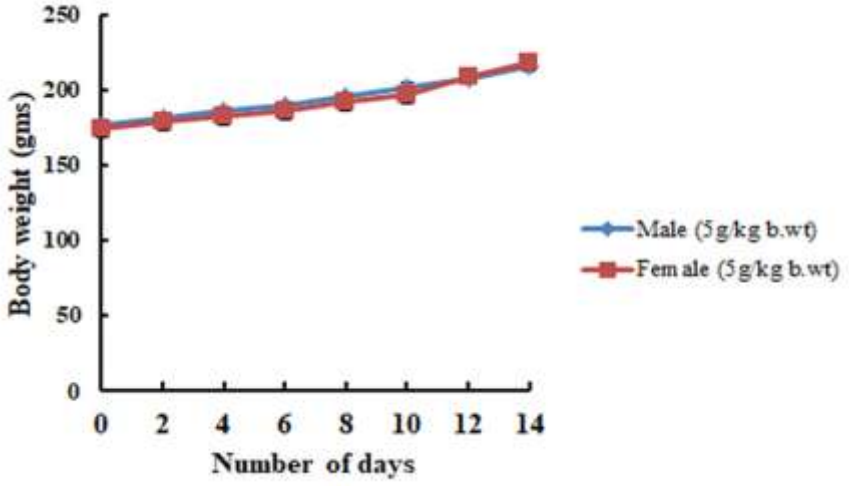

Figure 1:- Effect of DGMAF (5 gm/kg b.wt/day) on body weight of animal. Values are expressed as mean ± SD for six animals

Table 1:- Effect of administration of DGMAF (5 gm/kg b.wt/day) on hematological parameters. Values are expressed as mean ± SD for six animals.

Category	Hb (mg/dL)	WBC (mm^3)
Untreated	13.9±0.5	6542±358
Male	14.6±0.6	6833±551
Female	14.5±0.5	6333±503

Sub-acute toxicity:-
On oral administration of DGMAF for 60 days at doses of 100, 250 and 500 mg/kg b.wt, no mortality was seen in any of the groups. The animals were found to be generally healthy with no signs of toxicity during the observed

period of study. No abnormal changes in body weight (Figure 2) or relative organ weight (Table 2) and food consumption (Figure 3) were seen upon comparing with that of normal rats.

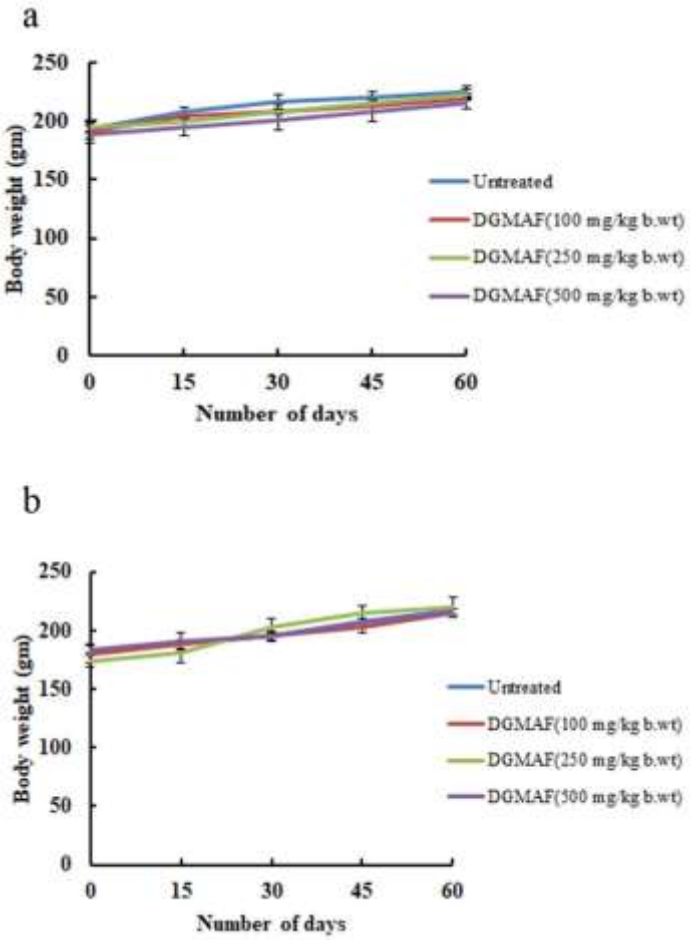

Figure 2:- Body weight of Wistar rats of either sex (a) Male (b) Female on administration of 100, 250 and 500 mg/kg b.wt of DGMAF. Values are expressed as mean ± SD for six animals

Table 2:- Effect of administration of DGMAF on relative organ weight of rat. Values are expressed as mean ± SD for six animals.

Untreated	Liver	Heart	Brain	Kidney	Spleen	Lungs	Stomach
Male	4 ± 0.3	0.4± 0.08	0.53± 0.04	0.2± 0.1	0.22± 0.1	0.4±0.08	0.3±0.1
Female	3.2±0.6	0.3± 0.07	0.53± 0.1	0.3± 0.02	0.25±0.02	0.32±0.02	03±0.12
DGMAF (100mg/kg b.wt)							
Male	3.4±0.7	0.3±0.05	0.6±0.05	0.2±0.02	0.2±0.07	0.3±0.14	0.3±0.06
Female	3.2±0.4	0.3±0.04	0.66±0.05	0.23±0.04	0.2±0.042	0.3±0.12	03±0.02
DGMAF (250mg/kg b.wt)							
Male	3.8±0.4	0.3±0.05	0.5±0.06	0.23±0.03	0.21±0.05	0.39±0.1	0.31±0.03
Female	3.1±0.2	0.4±0.03	0.49±0.2	0.2±0.02	0.2±0.03	0.4±0.14	0.3±0.1
DGMAF (500mg/kg b.wt)							
Male	4±0.8	0.32±0.12	0.54±0.3	0.43±0.05	0.25±0.031	0.38±0.07	0.24±0.1
Female	3.4±0.9	0.34±0.07	0.41±0.25	0.41±0.02	0.26±0.07	0.34±0.17	0.34±0.14

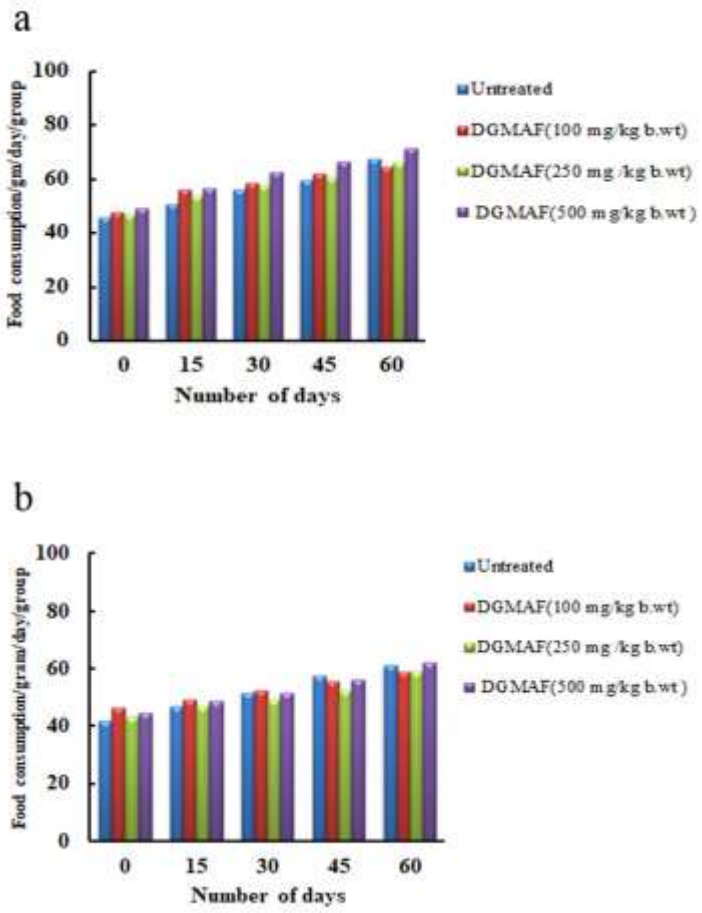

Figure 3:- Effect of administration of DGMAF on food consumption in (a) Male (b) Female Wistar rats.

Hematological parameters such as Hb level, total WBC count and differential counts were found to be within normal limits in all groups (Table 3). Similarly, biochemical parameters remained unaffected as the level of liver function tests (Table 4), kidney function tests (Table 5) were also within normal values having no abnormalities of statistical significance. Necroscopy of animals after 60 days did not produce any anatomical changes in rats.

Table 3:- Effect of DGMAF administration on hematological parameters. Values are expressed as mean ± SD for six animals.

Category	HB (g/dL)	RBC (million/cu-mm)	TC (Cell/cu-mm)	P (%)	L (%)	E (%)
Untreated						
Male	12.2± 0.4	4.2 ± 0.21	8210±244.1	37.5±2.1	56.5±4.7	3.5±0.7
Female	11.9± 0.15	4 ± 0.14	7495±324.07	46±1.4	54±4.8	4.5±0.6
DGMAF100 mg/kg b.wt)						
Male	13 ±2	4.7±1.2	13400±417.2	37±2.82	58.5±2.1	4.5±1.7
Female	14.7±2.2	5.2±1.3	14500±318.2	43±1.4	61.5± 5.7	4 ±1.1
DGMAF(250 mg/kg b. wt)						
Male	14.9±1.06	5.35± 2	11750±514.3	36±1.4	59.5± 3.7	5.5±0.7
Female	12.9±1.2	4.5 ± 1.1	12450±308.5	28±1.41	64 ±1.4	4 ±2.1
DGMAF(500 mg/kg b. wt)						
Male	12.2± 0.5	4.4 ± 0.9	12500±422.1	28.5±2.3	62.5±2.3	4.9±2.7
Female	12.4 ± 0.7	4.1± 0.8	9795±416.5	37±1.4	60.5±5.7	5 ± 1.4

Table 4:- Effect of DGMAF administration on liver enzymes of Wistar rats. Values are expressed as mean ± SD for six animals.

Category	Total bilirubin	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TP (IU/L)
Untreated Male	0.32±0.08	128±7.5	64±11	281±8	7.9± 1.5
Female	0.41±0.02	135±5.5	59±7.09	283±6	7±1.9
DGMAF(100 mg/kg b.wt)					
Male	0.3±0.08	144±3.4	71±3	263.6±10	7.3±2.5
Female	0.4±0.04	144±4.04	71±3.1	265±14	7.7±1.3
DGMAF(250 mg/kg b.wt)					
Male	0.45±0.04	145±4.1	70.3±5.5	266±9	7.4±1.4
Female	0.36±0.05	144±3.8	74±3.2	268±7.4	7.8±2.3
DGMAF(500 mg/kg b.wt)					
Male	0.42±0.05	129±8	62±7.2	266±15	7.9±2.4
Female	0.37±0.03	126±4	56±8	252±7.2	7.4±1.5

Table 5:- Effect of DGMAF administration on serum urea and creatinine levels in Wistar rats. Values are expressed as mean ± SD for six animals.

Category	Serum urea (mg/dL)	Serum creatinine (mg /dL)
Untreated		
Male	62±4.5	0.55±0.04
Female	58±8.4	0.53±0.03
DGMAF(100 mg /kg b.wt)		
Male	65±3.1	0.54±0.03
Female	58.4±2.4	0.47±0.02
DGMAF(250 mg /kg b.wt)		
Male	65±5.3	0.56±0.03
Female	60±1.2	0.47±0.02
DGMAF(500 mg /kg b.wt)		
Male	62±2.1	0.42±0.08
Female	56±4.1	0.46±0.04

Histological examination of organs such as spleen, liver, intestine and kidney of all the DGMAF treated groups did not show any differences when compared with untreated groups indicating that no adverse toxicological effects had occurred in these organs (Figure 4). In liver the hepatic architecture and arrangement of hepatocytes were not altered by DGMAF treatment and no change was observed compared to the normal group (Figure 4a). Appearance of glomerular tufts and tubules of kidney showed no differences between the treated and normal groups (Figure 4b). Spleen and intestinal villi also did not show any change in appearance (Figure 4c and 4d). No necrosis was observed in the histological study during the treatment period.

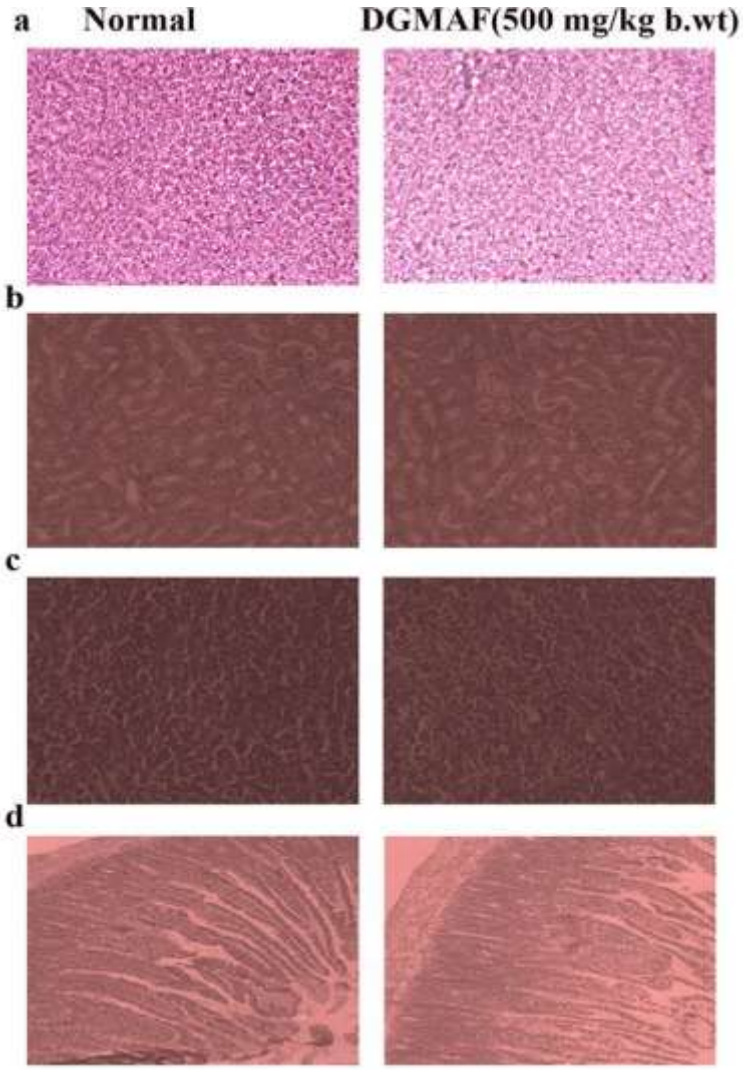

Figure 4:- Effect of sub-acute administration of DGMAF on histology of different organs of treated animals. (a) Liver (b) Kidney (c) Spleen (d) Intestine.

Discussion:-

Natural products therapies are supposed to be harmless and less injurious to the human body than the synthetic drugs (Alam *et al*., 2011). Some pharmacological and toxicological data exist for these types of natural medicines (Fragoso *et al*., 2008). However, their safety has repeatedly been probed due to reported complaints and fatality on the test animals (Park *et al*., 2010). So it is significant to determine the safety of these remedies in order to support their use.

Acute toxicity is an initial screening step, mandatory in the toxic assessment and evaluation of all biological compounds (Akhila *et al*., 2007) and it establishes the moderate lethal dose (LD_{50}) of biological compounds (Robinson *et al*., 2007). Acute toxicity is usually defined as the variations occurring instantly or a short time following a single administration of compound within 24 hrs (OECD, 2000).

In the present acute toxicity study, Wistar rats of both sexes were treated with DGMAF up to a dose of 5g/kg b.wt and no mortality or any signs of toxicity or side effects were recorded. In acute toxicity testing, doses higher than 5g/kg b.wt are generally not considered as dose related toxicity (Hayes, 1987). According to the OECD guidelines (Guidance Document for Acute Oral Toxicity Testing; OECD, 2001), compounds with LD_{50} values above 2 g/kg b.wt are generally considered to be relatively safe. Thus, DGMAF can be considered to be non-toxic at acute administration as the fractions are well tolerated and there has been no observed harmful effect.

In sub-acute toxicity study, following administration of DGMAF at 100, 250 and 500 mg/kg b.wt for 60 days, the body and organ weight are found to be normal without harming liver, blood or kidney tissue physiology or its tissue architecture. Change in organ-to-body weight ratio is the indication of organ damage (Busari *et al*., 2015). Comparison of organ weights between treated and control group of animals have conventionally been used to assess the toxic or harmful effects of drugs (Boyd, 1966 ; Pfeiffer, 1968). DGMAF does not have any harmful effects on rats that would cause them to loose appetite, thereby causing a reduction in food intake and subsequently a reduction in weight with increase in dose. Assessment of liver and kidney function is very important in evaluating the toxicity of plant extracts and an elevation in liver enzymes (ALT, AST and ALP) activity is conventionally an indicator of liver injury (Chavda *et al*., 2010). In the present study liver marker enzymes, blood urea and creatinine levels are found to be unaltered in DGMAF treated animals over the 60 days period.

Hematological changes such as anemia are often accompaniments of bone marrow toxicity and analysis of blood parameters with respect to animal studies have a high relevance and predictive value for humans (Hamid Rhiouani *et al*., 2008; Rhiouani *et al*., 2008). In this study hemoglobin and total differential count in the blood remained the more or less the same indicating the non-toxicity of DGMAF.

Conclusion:-

In conclusion, the present investigation demonstrates that at doses consumed in the traditional medicine, the aqueous fraction of methanolic extract of *D. gyrans* may be considered as relatively safe, as it does not cause either mortality or produce severe toxicological effects on selected body organs, biochemical indices and hematological markers of rats during the acute and sub-acute periods of study. These findings are very important as DGMAF with its hypolipidemic properties have the potential to be considered as a safer drug candidate for long term use in the treatment of atherosclerosis.

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