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ORA- Experimental Research

Evaluation of hepatoprotective activity of *Murvadi Agada* - A polyherbal formulation in Methotrexate induced hepatotoxicity in Wistar albino rats.

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ABSTRACT:

Background– In India, drug induced liver injury constitutes most cases of acute liver failure with fatality up to 50%. *Murvadi Agada* is a classical ayurvedic formulation indicated for the treatment of *Gara Visha* (concomitant poison); however, its hepatoprotective potential has not yet been scientifically evaluated. **Objective**- To evaluate the hepatoprotective effect of *Murvadi Agada* against Methotrexate induced hepatotoxicity using Wistar albino rats. **Methods**- Thirty Wistar albino rats of either sex were randomly divided into five groups (n=6). Group I served as normal control. Group II received single intraperitoneal injection of Methotrexate 20mg/kg and served as toxic control. Group III and IV were administered with *Murvadi Agada* (1080 and 2160 mg/kg) orally, respectively, followed by single intraperitoneal injection of Methotrexate. Group V received Silymarin (100 mg/kg orally) + Methotrexate. Silymarin and *Murvadi Agada* were administered for 7 and 14 consecutive days respectively. On the last day, single dose of Methotrexate was given one hour after drug administration. After 24 hours, the rats were euthanized to collect blood samples and liver tissues for assessing biochemical parameters, inflammatory markers and histopathological changes. **Results**- Methotrexate significantly increased serum AST, ALP, IL-1β ($p < 0.01$), GGT ($p < 0.05$) compared to normal control group. *Murvadi Agada* 1080 mg/kg significantly decreased serum ALT ($p < 0.001$), ALP ($p < 0.01$), AST ($p < 0.05$); *Murvadi Agada* 2160 mg/kg significantly decreased serum ALT, GLDH ($p < 0.001$), AST, ALP ($p < 0.01$), GGT, IL-1β ($p < 0.05$) and Silymarin significantly decreased serum IL-1β ($p < 0.01$), AST, GLDH ($p < 0.05$) compared to Methotrexate group. Single dose of Methotrexate caused areas of focal necrosis in liver sections; *Murvadi Agada* administration caused dose dependent reduction of inflammatory infiltrate in both groups and no histological changes were observed in Silymarin group. **Conclusion**– *Murvadi Agada* demonstrated significant hepatoprotective activity against Methotrexate induced hepatotoxicity in Wistar albino rats. Both doses significantly reduced liver enzymes, with the double therapeutic equivalent dose attenuating GGT, GLDH, IL-1β and histopathology protection comparable with Silymarin.

KEYWORDS: *Gara Visha,* hepatoprotective activity, hepatotoxicity, Methotrexate, *Murvadi Agada*

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1. INTRODUCTION

The liver is considered to be a vital organ that functions as a central hub of metabolism for proteins, carbohydrates, lipids and simultaneously performing the excretion of waste metabolites. Its primary defense role involves biotransformation to detoxify and excrete both endogenous waste and foreign substances (xenobiotics) into the bile. Any failure in this complex detoxification capacity renders the liver highly vulnerable to injury, making it the central target for conditions like drug induced liver injury (DILI). [1] It is observed that due to abundant usage of newly developed drugs (e.g. monoclonal antibodies for auto immune diseases, immune checkpoint inhibitors for cancer) and the tendency to consume dietary supplements, cases of DILI are likely to increase which attracts international interest. The incidence of DILI is difficult to decide and is mostly underestimated. Diagnosis depends upon the exclusion of all other cases of liver injury which makes it difficult for accurate diagnosis. Clinical manifestations can range from asymptomatic elevation of liver enzymes to outcomes such as liver failure, transplantation or death following the onset of DILI. Most patients recover after discontinuing the drugs, but some progress to fatal acute liver failure. [2]

Yakrut (liver) is the seat *for Ranjaka Pitta* (subtype of *Pitta*) which transforms *Rasa* (nutrients) into *Rakta* (blood). Since it mainly performs the biological functions of metabolism and detoxification, disruption in its functional equilibrium renders it vulnerable to *Roga* (diseases). *Gara Visha* (concomitant poison) results from the combination of low-potency toxins from animate sources and substances with diametrically opposite physiological actions. [3] It is therefore correlated with contemporary conditions like drug induced liver injury (DILI). The primary action of this slow-acting poison is to suppress *Pavaka* (digestive fire), leading to a state of *Mandagni* (low metabolic activity). *Yakrut Vikara* (liver disorders) is a common manifestation that may develop due to the slow acting toxicity of *Gara Visha* (concomitant poison). [4] *Murvadi Agada* mentioned in *Ashtanga Hridaya, Visha-Pratishedha* is a polyherbal formulation which is indicated in *Gara Visha* concomitant poison). It is composed of *Murva* (*Marsdenia tenacissima* (Roxb.) Moon), *Guduchi* (*Tinospora cordifolia* (Thunb.) Miers), *Tagara* (*Valeriana wallichii* DC.), *Pippali* (*Piper longum* L.), *Patola* (*Trichosanthes cucumerina* L.), *Chavya* (*Piper retrofractum* Vahl), *Chitraka* (*Plumbago zeylanica* L.), *Vacha* (*Acorus calamus* L.), *Musta* (*Cyperus rotundus* L.) and *Vidanga* (*Embelia ribes* Burm. f.) plants. *Murvadi Agada* which acts on *Garopahata Pavaka* (effect of concomitant poisons on the digestive fire) helps in boosting the metabolism, and substantially cure hepatic injury. [5] The scientific validation regarding the hepatoprotective activity of *Murvadi Agada* is not yet explored.

Methotrexate (MTX), a folate antagonist, is used as an anticancer drug for diseases such as leukemia, breast cancer, and osteosarcoma. It is additionally prescribed for inflammatory conditions including rheumatoid arthritis, psoriasis, and Crohn's disease. However, research indicates that MTX can cause hepatotoxicity, leading to tissue injury, impaired liver function, cirrhosis, and fibrosis, along with changes in liver enzyme levels and liver histology. [6] In the current scenario, treatment of DILI is limited to supportive care and few available antidotes which highlights the need of an effective hepatoprotective drug. The individual drugs of *Murvadi Agada* are known to possess hepatoprotective effects but their combined effect has yet to be studied. Considering this fact, the present study design is planned to assess the hepatoprotective activity of *Murvadi Agada* against MTX induced hepatotoxicity in Wistar albino rats. Since Silymarin is the most frequently used natural compound for the treatment of hepatic diseases worldwide due to its anti-

oxidant, anti-inflammatory, and anti-fibrotic activities, in the present study, it was taken as a standard drug to compare the efficacy of *Murvadi Agada* against MTX induced hepatotoxicity.

2. MATERIALS METHODS

Plant materials: Most of the raw drugs of *Murvadi Agada* were collected from the Pharmacy, ITRA, Jamnagar, Gujarat. *Murva, Patola* and *Chitraka* samples were collected from Narayan Aushadha Bhandar, Jamnagar, Gujarat. As per API, drugs which are used in the finished product of *Murvadi Agada* were identified and authenticated by the Pharmacognosy Laboratory, ITRA, Jamnagar, Gujarat. All the ten ingredients were taken in equal quantity, dried and made into very fine powder separately. These individual fine powders obtained were sieved through 80 no. mesh. All the drugs were mixed homogenously for more than seven times. The homogenously mixed fine powder was packed in airtight containers. [7]

Experimental animals: Thirty Wistar albino rats (*Rattus norvegicus*) of either sex (12 males, 18 females), weighing 150–250 g and aged 8–10 weeks, were procured from the institutional animal house attached to S.D.M. Centre for Research in Ayurveda and Allied Sciences, Udupi, Karnataka and included in the study following random selection. Animals were maintained under controlled conditions of temperature (23 ± 2 °C) and humidity (50–60%) with natural light–dark cycles. Standard commercial pellet diet and water were made available without restriction throughout the experimental period. Only healthy, treatment-naive animals with normal behavior were included. All animals were acclimatized one week before the experiment. The study was conducted in accordance with CCSEA guidelines, after approval from the Institutional Animal Ethics Committee (SDMCRA/IAEC/IT-J-A-15, dated 16/09/2024). No animals were excluded during the study.

Experimental study design: Five groups of animals were designed for the trial, with each group consisting of six rats. The dose of *Murvadi Agada* was calculated by converting the human therapeutic dose to rat dose based on body surface area conversion factor as per Paget and Barnes, [8] which yielded a therapeutic equivalent dose of 1080 mg/kg body weight. The double therapeutic equivalent dose was taken as 2160 mg/kg. The powder was administered orally with distilled water using gastric cannula according to the body weight of albino rats.

In Group I (normal control), animals received normal diet and water *ad libitum*. In Group II (MTX control), animals received distilled water per os (P.O.) for 14 days and a single intraperitoneal (I.P.) injection of MTX (IPCA, Mumbai) (20 mg/kg) on the 14th day. [6] Group III and IV were administered with *Murvadi Agada* (1080 and 2160 mg/kg) orally, respectively for 14 consecutive days. In both groups, a single intraperitoneal (I.P.) injection of MTX (20 mg/kg) was administered 1 hour after the test drug on the 14th day. In Group V (Standard), animals received Silymarin (Micro Labs Ltd, Bengaluru) (100 mg/kg P.O.) for 7 consecutive days, and a single intraperitoneal (I.P.) injection of MTX (20 mg/kg) was administered 1 hour after the standard drug on the 7th day.

Blood and tissue sampling: After 24 hours of MTX administration, animals were briefly anesthetized by di-ethyl ether inhalation, and blood was collected in a plain vacutainer tube (red-top) from the retro-orbital plexus. The blood was kept in a centrifuge tube and later centrifugation was performed at 3000 rpm at 30°C for 20 min to separate serum for estimation of various biochemical parameters and inflammatory factor IL-1β. After blood collection, the anesthetized animals were euthanized using carbon dioxide (CO_2) inhalation in a euthanasia chamber. CO_2 was introduced gradually to achieve a displacement rate of approximately 20–30% of the chamber volume per minute, ensuring minimal

distress. Animals were monitored until cessation of respiration and death was ensured by absence of heartbeat and reflexes for minimum 5 minutes before sample collection. The liver was carefully dissected out, cleaned to remove adherent tissues, gently blotted to eliminate residual blood and weighed. A portion of liver tissue was fixed in 10% buffered formalin solution for histopathological evaluation.

Serum biochemical analysis: Liver function biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin were determined according to the methods of Reitman and Frankel, King and Armstrong, Malloy and Evelyn respectively using standard assay kits. [9] [10] [11] Total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL) were assessed by enzymatic colorimetric methods. Blood urea, uric acid and creatinine were measured by the classical Diacetyl Monoxime (DAM), Caraway and Jaffe methods. [12] [13] [14] Gamma-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GLDH) and lactate dehydrogenase (LDH) were estimated according to the methods of Szasz, Schmidt et al, Wroblewski and LaDue. [15] [16] [17]

Cytokine IL-1β measurement: Following centrifugation, the supernatant was obtained and the quantification of Interleukin – 1 beta (IL-1β) in the serum was done using a sandwich Enzyme linked immunosorbent assay (ELISA) method employing the Rat IL-1β ELISA kit from ELK Biotech (cat. no. ELK1272); sensitivity: 5.9 pg/mL; detection range: 15.63–1000 pg/mL. The microtiter plate provided in the kit was pre coated with antibody specific to Rat IL-1β. Serum samples (100 µL) were added to appropriate wells and incubated at 37°C for 80 minutes, followed by incubation with a biotin- conjugated anti-rat- IL-1β antibody (100 µL) at 37°C for 50 minutes. Subsequently, Streptavidin conjugated to Horseradish Peroxidase (Streptavidin – HRP, 100 µL) was added and incubated at 37°C for 50 minutes. After washing, 90 µL of TMB (3,3’,5,5’-Tetramethylbenzidine) substrate solution was introduced and incubated at 37°C for 20 minutes under dark conditions. The enzyme- substrate reaction was terminated by the addition of 50 µL of sulfuric acid as Stop Reagent, which yielded a color change from blue to yellow. Optical density (OD) was recorded spectrophotometrically at 450 nm using a microplate reader and the IL-1β concentrations in each sample were assessed by comparing the OD values against a standard curve generated from serially diluted standards (15.63–1000 pg/mL). All steps were carried out in strict accordance with the manufacturer’s instructions.

Histopathology of liver tissues: Liver tissues were subjected to fixation in 10% neutral buffered formalin, processing in ascending grades of alcohol**,** clearing in xylene and embedding in paraffin wax. Tissue sections of 5µm thickness were made, subjected to Hematoxylin and Eosin (H&E) staining and examined. The photomicrographs were captured using an Olympus CX23 light microscope equipped with an Olympus SC50 digital camera at 100x magnification. Histopathological evaluation was performed qualitatively by assessing features such as hepatocellular degeneration, necrosis, inflammatory infiltration, and architectural changes.

Statistical analysis: Data were presented as mean ± standard error of mean (SEM; n=6 per group). Prior to analysis, data distribution was assessed using the Shapiro–Wilk normality test and homogeneity of variances was confirmed by Levene's test. Inter-group comparisons were performed using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test to compare all treatment groups against the Methotrexate control group. $p<0.05$ was regarded as statistically significant. Statistical analysis was conducted using Sigma Stat software, version 3.5.

3. RESULTS

Gross observations in hepatoprotective study: During the experimental period, all the animals were found to be healthy without any changes in normal behavior or activity. The liver collected from normal control group was bright red in color and from MTX control group was pale in color. The livers collected from *Murvadi Agada* therapeutic equivalent dose group, *Murvadi Agada* double therapeutic equivalent dose group and Silymarin group were red in color.

Serum biochemical parameters: Administration of MTX showed significant increase in serum AST, ALP, IL-1β ($p < 0.01$), GGT ($p < 0.05$) with significant decrease in serum HDL, VLDL, serum triglycerides ($p < 0.001$), serum cholesterol, serum creatinine ($p < 0.01$) when compared with normal control group (Table 1, Table 2). There was a significant decrease in serum ALT ($p < 0.001$), ALP ($p < 0.01$) and AST ($p < 0.05$) with significant increase in serum cholesterol, HDL, serum creatinine ($p < 0.01$) and serum triglycerides, VLDL ($p < 0.05$) in *Murvadi Agada* therapeutic equivalent dose group compared with the MTX control group (Table 1). There was a significant decrease in serum ALT, GLDH ($p < 0.001$), AST, ALP ($p < 0.01$) and GGT, IL-1β ($p < 0.05$) with significant increase in serum creatinine, serum urea ($p < 0.01$) in *Murvadi Agada* double therapeutic equivalent dose group compared with the MTX control group (Table 1, Table 2). In the Silymarin group, there was significant decrease in serum IL-1β ($p < 0.01$) and AST, GLDH ($p < 0.05$) with significant increase in serum creatinine, serum urea ($p < 0.001$) compared with the MTX control group (Table 1, Table 2). Total bilirubin levels were raised in MTX control group compared to normal control group. On the contrary, total bilirubin levels were decreased in both *Murvadi Agada* treated groups and Silymarin treated group compared with MTX control group. None of these was found to be statistically significant (Table 1).

Ponderal changes: MTX control group showed significant reduction in body weight ($p < 0.001$) in comparison with normal control group. *Murvadi Agada* therapeutic equivalent dose group, *Murvadi Agada* double therapeutic equivalent dose group and Silymarin group showed minimal weight loss in comparison with MTX control group but this was not statistically significant. Liver weight showed significant increase ($p < 0.01$) in MTX control group in comparison with normal control group. *Murvadi Agada* therapeutic equivalent dose group showed decrease in liver weight in comparison with MTX control group which was not statistically significant. Also, *Murvadi Agada* double therapeutic equivalent dose group and Silymarin group displayed increase in liver weight in comparison with MTX control group which was not statistically significant (Table 3).

Histopathological changes: The histopathological report of all groups showed lobular arrangement of liver tissue. Each lobule consisted of a central vein and portal triads along the periphery of lobules. Numerous sinusoids pass radially from central vein and the spaces between the sinusoids contain liver cells. The normal control group showed normal hepatic cytoarchitecture with intact hepatocytes and central vein. [Fig 1 (a)]. In MTX control group, areas of focal necrosis showing loss of cellular and nuclear details accompanied by infiltration of acute and chronic inflammatory cell were seen [Fig 1 (b)]. Mild focal portal chronic lymphocytic infiltrate was also observed [Fig 1 (c)]. In *Murvadi Agada* therapeutic equivalent dose group, mild focal portal chronic lymphocytic infiltrate was seen in few areas [Fig 1 (d)]. In *Murvadi Agada* double therapeutic equivalent dose group, focal chronic inflammatory lymphocytic infiltrate was seen in one area [Fig 1 (f)]. In both *Murvadi Agada* therapeutic equivalent dose group and *Murvadi Agada* double therapeutic equivalent dose group, degeneration was not seen. No changes were observed in tissue architecture. Also, compared with MTX

control group, absence of necrosis was noted [Fig. 1(e), Fig. 1(g)]. Histological changes like inflammation, degeneration and necrosis were not seen in Silymarin group. Compared with MTX control group, there was absence of necrosis and inflammatory infiltrate. Also, no changes in tissue architecture were observed. [Fig. 1(h)].

Table 1: Effects of *Murvadi Agada* on serum biochemical parameters in MTX induced hepatotoxicity in Wistar albino rats

Sl. No	Parameters	Normal control	MTX (20 mg/kg I.P.)	*Murvadi Agada* (1080 mg/kg P.O.) + MTX	*Murvadi Agada* (2160 mg/kg P.O.) + MTX	Silymarin (100 mg/kg P.O.) + MTX
1	AST (IU/L)	116.66 ± 6.74	145.83 ± 5.87 $^{**\&\&}$	125.83 ± 4.68 $^{@\$}$	112 ± 2.34 $^{@@\$\$}$	127.5 ± 2.43 $^{@\$}$
2	ALT (IU/L)	55.83 ± 1.44	62.16 ± 3.46	40.66 ± 2.77$^{@@\$\$\$}$	36.6 ± 3.04$^{@@\$\$\$}$	53 ± 3.15
3	ALP (IU/L)	494.33 ± 18.28	760.33 ± 77.54$^{**\&\&}$	397.5 ± 51.95$^{@@\$\$}$	419.2 ± 40.3$^{@@\$\$}$	632.83 ± 59.02
4	Total bilirubin (mg/dl)	0.183 ± 0.030	0.188 ± 0.026	0.155 ± 0.017	0.144 ± 0.019	0.17 ± 0.029
5	Direct bilirubin (mg/dl)	0.043 ± 0.012	0.051 ± 0.010	0.058 ± 0.007	0.042 ± 0.009	0.053 ± 0.011
6	Indirect bilirubin (mg/dl)	0.14 ± 0.0216	0.136 ± 0.0217	0.096 ± 0.014	0.102 ± 0.013	0.116 ± 0.018
7	Serum urea (mg/dl)	48.83 ± 3.46	45.66 ± 1.25	49.83 ± 3.02	57.2 ± 3.83 $^{@\$\$}$	59.5 ± 1.05$^{@@\$\$\$}$
8	Serum uric acid (mg/dl)	1.35 ± 0.076	1.45 ± 0.095	1.55 ± 0.114	1.58 ± 0.115	1.416 ± 0.094
9	Serum creatinine (mg/dl)	0.546 ± 0.016	0.486 ± 0.008$^{\&\&}$	0.66 ± 0.023 $^{@@\$\$}$	0.63 ± 0.027$^{@@\$\$}$	0.616 ± 0.028$^{@@\$\$\$}$
10	Serum cholesterol (mg/dl)	53.83 ± 3.4	38.66 ± 2.97$^{*\&\&}$	57.66 ± 4.77$^{@@\$\$}$	46.2 ± 3.78	37 ± 2.95
11	Triglycerides (mg/dl)	145.16 ± 10.85	74.5 ± 5.1$^{**\&\&\&}$	98 ± 7.44$^{\$}$	62.2 ± 9.1	65.16 ± 3.62
12	HDL (mg/dl)	29.5± 1.70	16.5 ± 0.95$^{**\&\&\&}$	26.66 ± 2.17 $^{@@\$\$}$	20.2 ± 2.81	16.83 ± 1.4
13	LDL (mg/dl)	8.96 ± 2.43	8.43 ± 1.95	11.4 ± 2.78	13.56 ± 1.44	13.03 ± 3.49
14	VLDL (mg/dl)	29.03 ± 2.17	15.06 ± 0.98$^{**\&\&\&}$	19.6 ± 1.48$^{\$}$	12.44 ± 1.82	13.03 ± 0.72

(Data represented in Mean ± SEM; n = 6) $^{\&\&\&}p$<0.001 when compared to normal control group, $^{\&\&}p$<0.01 when compared to normal control group; $^{\$\$\$}p$<0.001 when compared to MTX control, $^{\$\$}p$<0.01 when compared to MTX control, $^{\$}$ p<0.05 when compared to MTX control (Unpaired 't' test); **p<0.01 when compared to normal control group, *p<0.05 when compared to normal control group; $^{@@}$ p<0.01 when compared to MTX control, $^{@}p$<0.05 when compared to MTX control (ANOVA followed by Dunnett's multiple 't' test). MTX: Methotrexate; P.O.: per os; I.P.: intraperitoneal.

Table 2: Effects of *Murvadi Agada* on liver specific biomarkers in MTX induced hepatotoxicity in Wistar albino rats

Sl. No	Parameters	Normal control	MTX (20 mg/kg I.P.)	*Murvadi Agada* (1080 mg/kg P.O.) + MTX	*Murvadi Agada* (2160 mg/kg P.O.) + MTX	Silymarin (100 mg/kg P.O.) + MTX
1	GGT (IU/L)	1.083 ± 0.314	2.1 ± 0.291*	1.5 ± 0.235	1.1 ± 0.144$^{@}$	1.3 ± 0.238
2	GLDH (IU/L)	0.117 ± 0.017	0.118 ± 0.007	0.148 ± 0.012	0.073 ± 0.04 $^{@\$\$\$}$	0.090 ± 0.004$^{\$}$
3	LDH (IU/L)	450.66 ± 48.46	611.66 ± 89.45	727.16 ± 111.64	514.4 ± 24.77	542.33 ± 43.32
4	IL–1β (pg/mL)	15.19 ± 2.163	30.95 ± 4.732 $^{**\&}$	20.15 ± 2.146	15.38 ± 3.802$^{@\$}$	12.94 ± 0.538 $^{@@}$

(Data represented in Mean ± SEM; n = 6) $^{\&}p$<0.05 when compared to normal control group; $^{\$\$\$}p$<0.001 when compared to MTX control, $^{\$}$ p<0.05 when compared to MTX control (Unpaired 't' test); **p<0.01 when compared to normal control group, *p<0.05 when compared to normal control group; $^{@@}$ p<0.01 when compared to MTX control, $^{@}p$<0.05 when compared to MTX control (ANOVA followed by Dunnett's multiple 't' test). MTX: Methotrexate; P.O.: per os; I.P.: intraperitoneal.

Table 3: Effects of *Murvadi Agada* on ponderal changes in MTX induced hepatotoxicity in Wistar albino rats

Sl. No	Parameters	Normal control	MTX (20 mg/kg I.P.)	*Murvadi Agada* (1080 mg/kg P.O.) + MTX	*Murvadi Agada* (2160 mg/kg P.O.) + MTX	Silymarin (100 mg/kg P.O.) + MTX
1	Body weight (g)	8.09 ± 0.768	-2.245 ± 0.280$^{**\&\&\&}$	-0.893 ± 0.778	-0.062 ± 1.682	-1.745 ± 1.23
2	Liver weight (g)	3.725 ± 0.171	5.45 ± 0.408 $^{*\&\&}$	5.385 ± 0.363	5.585 ± 0.160	5.7 ± 0.503

(Data represented in Mean ± SEM; n = 6) $^{\&\&\&}p$<0.001 when compared to normal control group, $^{\&\&}p$<0.01 when compared to normal control group (Unpaired 't' test); $^{**}p$<0.01 when compared to normal control group, $^{*}p$<0.05 when compared to normal control group (ANOVA followed by Dunnett's multiple 't' test). MTX: Methotrexate; P.O.: per os; I.P.: intraperitoneal.

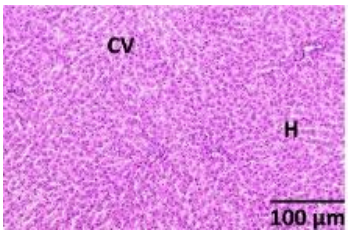

Fig 1 (a) – Liver section of rats belonging to normal control group with normal tissue architecture showing intact hepatocytes (H) and central vein (CV).

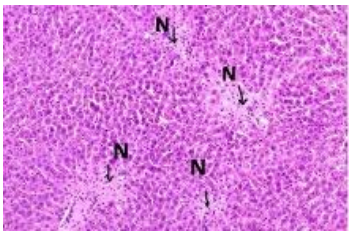

Fig 1 (b) - Liver section of rats belonging to Methotrexate control group showing focal necrosis (N) which are marked with black arrows.

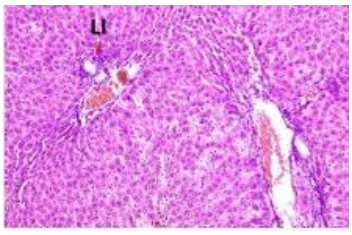

Fig 1 (c) - Liver section of rats belonging to Methotrexate control group showing mild focal portal chronic lymphocytic infiltration (LI) which are marked with red arrows.

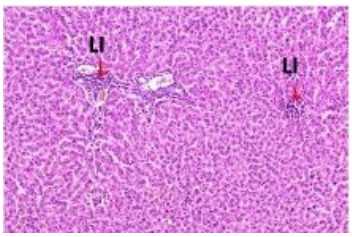

Fig 1 (d) - Liver section of rats belonging to *Murvadi Agada* therapeutic equivalent dose group showing mild focal portal chronic lymphocytic infiltration (LI) which are marked with red arrows.

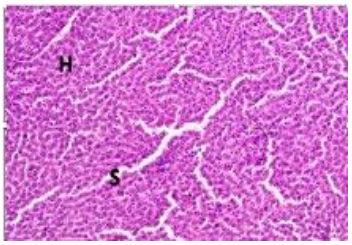

Fig 1 (e) – Liver section of rats belonging to *Murvadi Agada* therapeutic equivalent dose group showing areas with reduced histological changes with intact hepatocytes (H) and sinusoids (S).

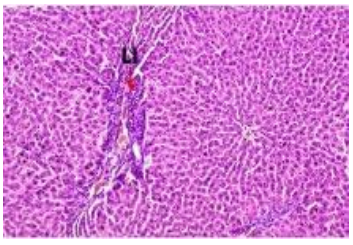

Fig 1 (f) - Liver section of rats belonging to *Murvadi Agada* double therapeutic equivalent dose group showing inflammatory infiltrate (LI) which are marked with red arrows.

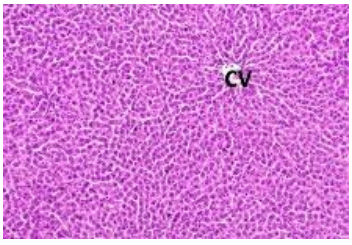

Fig 1 (g) Liver section of rats belonging to *Murvadi Agada* double therapeutic equivalent dose group showing areas with reduced histological changes with intact central vein (CV).

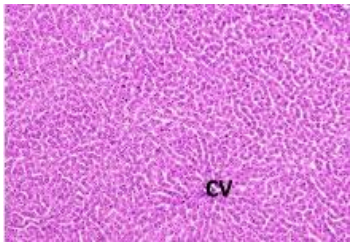

Fig 1 (h) – Liver section of rats belonging to Silymarin group showing normal histology with intact central vein (CV).

Figure 1: Photomicrographs of rat liver sections in different experimental groups (at 100x magnification)

4. DISCUSSION

The present study was undertaken to evaluate the hepatoprotective potential of *Murvadi Agada* against MTX induced hepatotoxicity in Wistar albino rats. *Gara Visha* (concomitant poison) being *Kalantaravipaki* (slowly manifesting toxicity), deranges the metabolism of the body and by virtue of its effect, the symptoms can be correlated to contemporary DILI. A*gnimandya* (diminished digestive fire) is the primary pathological event which progresses to conditions like *Pandu* (anaemia), *Kasa* (cough), *Swasa*

(dyspnoea)*, Yakrut Vikara* (liver disorders) *etc.* Considering the liver's indispensable role in metabolism and detoxification, it becomes the target organ in the pathogenesis of diseases due to *Gara Visha* (concomitant poison). *Murvadi Agada* rectifies *Garopahata Pavaka* (effect of concomitant poisons on the digestive fire) which subsequently repairs the disrupted hepatocellular metabolic integrity. The formulation consists of 10 herbal drugs that are predominantly *Katu* (pungent taste)*, Tikta* (bitter taste) *Kashaya Rasa* (astringent taste) and *Vata Kapha Hara.* The drugs are majorly *Ruksha* (dry)*, Tikshana* (sharp/penetrating) and *Laghu Guna* (light property*)*. The *Veerya* (potency) is predominantly *Ushna* (hot), and *Vipaka* (post – digestive effect) is considered to be *Katu* (pungent). [7]

Rats in MTX control group showed a decline in body weight that must have occurred due to reduced food intake, metabolic stress and systemic toxicity. On the other hand, both the groups treated with *Murvadi Agada* (1080 and 2160 mg/kg) maintained body weight effectively. Hepatomegaly observed in MTX control group was minimum in the *Murvadi Agada* therapeutic equivalent dose group. Among the biochemical parameters, rise in serum liver enzymes AST and ALT indicates damage to the hepatocytes with cellular leakage and loss of functional integrity of cell membrane in MTX control group. [18] *Murvadi Agada* treatment (1080 and 2160 mg/kg) produced a statistically significant reduction in both enzymes which reflects membrane stabilization and prevented hepatocyte necrosis. ALP which suggests hepatobiliary function and ductal integrity was also elevated in MTX control group. [19] Restoration of ALP in both *Murvadi Agada* treated groups indicate that the liver's secretory function is improving. MTX administration resulted in significant reduction in serum triglycerides, HDL, VLDL and serum cholesterol levels. Severe acute hepatocellular injury impairs the liver's capacity to synthesize and assemble lipoproteins, resulting in decreased lipid levels. [20] *Murvadi Agada* therapeutic equivalent dose group demonstrated significant increase in serum triglycerides, HDL, VLDL and serum cholesterol levels, indicating recovery of hepatic lipid synthetic functions. These effects can be attributed to the activity of drugs possessing *Deepana* (improves the digestive fire) *Pachana* (digests the Ama) action and *Katu Rasa* (pungent taste) such as *Pippali, Chavya, Chitraka, Vacha and Vidanga.* [21] The overall improvement is seen in gastro intestinal motility, enhancement of anti-oxidant defense system and reduction of inflammation.

The changes seen in GGT and GLDH provide more interpretive depth. GGT is an index of hepatic oxidative stress and its elevation in MTX control indicate biliary disease. [19] Reduction in GGT especially in the *Murvadi Agada* double therapeutic equivalent dose group implies anti-oxidant protection. GLDH is a specific signal of mitochondrial membrane rupture. Elevation of GLDH in MTX control group suggests advanced hepatocyte death. [22] Drugs predominant in *Kashaya Rasa* (astringent taste) such as *Musta* and *Tagara* acts as *Ropana* (tissue healing) and *Sangrahana* (retaining or holding). [23] It helps in mitochondrial membrane protection which is validated by significant decrease in GLDH levels in *Murvadi Agada* double therapeutic equivalent dose group.

Accumulation of MTX – Polyglutamate (MTX – PG) in hepatocytes induces oxidative stress by depleting glutathione and increasing lipid peroxidation, which activates the NF-κB signaling pathway, resulting in elevated IL-1β levels. [24] The significant elevation of IL-1β observed in MTX control group is consistent with the pro – inflammatory mechanism of MTX induced hepatotoxicity. The significant reduction of IL-1β in *Murvadi Agada* double therapeutic equivalent dose group can be attributed to the anti-inflammatory action of the constituent drugs such as *Guduchi* and *Patola* which are *Tikta*

Rasa (bitter taste), and manifests *Jwaraghna* (antipyretic) and *Kleda Shoshana* (removes excessive moisture) action. [25]

Histopathological findings are the most direct findings of hepatoprotection. While MTX control group demonstrated hepatocyte degeneration and necrosis, *Murvadi Agada* therapeutic equivalent dose group showed focal chronic lymphocyte infiltrate in few areas without any degeneration or necrosis. This indicates that even when inflammatory activity was persistent, cell death was prevented. In the *Murvadi Agada* double therapeutic equivalent dose group, inflammation was confined to one area suggesting dose dependent hepatoprotection.

The hepatoprotective effects of *Murvadi Agada* can be attributed to the combined action of bioactive phytochemicals present in the drugs. Kumar S et al. reported that Piperine, contributed by *Pippali* and *Chavya* decreases lipid peroxidation and upregulates the anti-oxidant defence system while amplifying intracellular anti-oxidant status by improving the membrane permeability to facilitate intracellular glutathione absorption. [26] Moreover, Pradeep CR and Kuttan G reported that Piperine inhibits NF-κB activation and subsequent pro – inflammatory cytokine production. [27] Sharma et al. reported that β-sitosterol, tinosporin, and palmatine found in *Guduchi* enhances endogenous antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase. [28] Kuan-Hong W and Bai-Zhou L reported that plumbagin, the principal constituent of *Chitraka* attenuates oxidative stress and inflammation by modulating the NF-κB and Nrf2 pathway. [29] Hei X et al. reported that β-asarone contributed by *Vacha* regulates the Nrf2/HO-1 pathway, thereby activating the anti-oxidant defense mechanism. [30] Qin X et al. reported that Embelin present in *Vidanga*, upregulates cellular anti-oxidant defense through activation of Nrf-2/Ho-1 pathway. [31] Dai S et al. reported that Cucurbitacin B, a major constituent of *Patola* suppresses the expression of pro inflammatory cytokines by inhibiting NF-κB activation. [32] Saha S et al. reported that phytochemicals present in *Murva* significantly increase superoxide dismutase activity and reduces malondialdehyde content in liver homogenates indicating anti-oxidant and hepatoprotective properties. [33]

The Silymarin group demonstrated significant reduction in serum AST, GLDH and IL-1β, along with complete preservation of liver architecture with no inflammation, no degeneration and no necrosis. The hepatoprotective efficacy of the double therapeutic equivalent dose of *Murvadi Agada* aligns with that of the standard drug Silymarin.

5. CONCLUSION

The present study demonstrated that *Murvadi Agada* exerts significant hepatoprotective activity against methotrexate induced hepatotoxicity in Wistar albino rats. Both tested doses of *Murvadi Agada* (1080 and 2160 mg/kg) effectively attenuated hepatocellular injury, as evidenced by significant reduction in serum liver enzymes AST, ALT and ALP. Moreover, *Murvadi Agada* therapeutic equivalent dose restored lipid alterations including serum cholesterol, serum triglycerides, HDL and VLDL. Notably higher dose demonstrated additional benefits, including significant reductions in GGT, GLDH, and the pro-inflammatory cytokine IL-1β parameters indicating anti-oxidant, anti-inflammatory activity and mitochondrial membrane protection. The histopathological examination revealed dose dependent hepatoprotection with complete absence of necrosis and degeneration in both *Murvadi Agada* treated groups. The *Murvadi Agada* double therapeutic equivalent dose group was comparable to Silymarin group. Hence, overall findings support the hepatoprotective potentials of *Murvadi Agada.* Further studies including chronic toxicity assessments and clinical trials in patients with drug induced liver injury are warranted to establish its safety and therapeutic applicability.

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