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EVALUATION OF ANTI-CANCER ACTIVITY OF VASAGULUCHYADI KASHAYAM ON DEN AND PHENOBARBITONE INDUCED HEPATOCELLUAR CARCINOMA IN MALE RATS

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ABSTRACT

The objective of this study is to find the chemoprotective effect of Vasaguluchyadi Kashayam in DEN and PHENOBARBITONE induced hepatocarcinogenesis in male Sprague Dawley rats. The important steps involved selection, constituent Analysis, *in-vivo* and *in-vitro* antioxidant activity. Initiation and promotion of Hepatocellular carcinoma (HCC) was done by single i.p injection of DEN at a dose of 200mg/kg and phenobarbitone 0.05% was given through orally in drinking water. The standard [5-flurouracil (20mg/kg) i.p] and formulation treatment were given after 14 days of development of HCC. The results showed that the injection DEN and PHENOBARBITONE (through drinking water) lead to the development of liver tumors in rats. In disease control group, serum biochemical and haemotological parameters such as SGOT, SGPT, ALP, urea, total bilirubin, total protein and creatinin levels were increased, and RBC, WBC and Hb levels were decreased when compared to normal group. In disease control group SOD, CAT, GSH levels were decreased and LPO level was increased. The result revealed that formulation treatment (Preventive) group gives excellent shielding against HCC and produced all the parameter in near normal range with a maintained antioxidant enzyme system. The results obtained showed that formulation was found to containing phenols at a concentration of 52.06 mg/g and flavonoids 67.84 mg/g. In the DPPH, ABTS, FRAP and phosphomolybdenum assay formulation has produced the highest antiradical activity and was also comparable with the standard quercetin The present study reveal the

efficacy of the Formulation to prevent malignancy induced by chemical carcinogen and the constituents responsible for activity.

KEYWORDS: DPPH, ABTS, FRAP and phosphomolybdenum.

INTRODUCTION

Cancer is a disease in which cells in the body grow out of control. Cells in nearly any part of the body can become cancer, and can spread to other areas of the body. These contrast with benign tumors, which do not spread to other parts of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. Sometimes, however, cells become abnormal and keep dividing to form more cells without control or order, creating a mass of excess tissue called a tumor. Tumors can be cancerous or non cancerous.[1] The cells in malignant tumors can invade and damage nearby tissue and organs. Hepatocellular carcinoma (HCC) is the main form of primary liver cancer and is histologically and etiologically distinct from other forms of primary liver cancer. They are either primary when the cancer starts in itself, or metastatic, when the cancer has spread to liver from some other part of the body.[2] Approximately 70%–90% of patients with HCC have an established background of chronic liver disease and cirrhosis, with major risk factors for developing cirrhosis including chronic infection with Hepatitis B virus (HBV), Hepatitis C virus (HCV), alcoholic liver disease, and NASH. Other risk factors for developing HCC include intake of aflatoxin-contaminated food, diabetes, obesity, certain hereditary conditions such as hemochromatosis, and some metabolic disorder**.** Ayurvedic formulation such as Vasaguluchyadi Kashayam plays an important role in human healthcare. The formulation is used for treating several ailments like Anaemia, Jaundice, Liver complications such as fatty liver, Haemorrhage, Rheumatoid Arthritis and Bleeding Problems.[3] DEN (diethyl nitrosamine) is a well known hepatocarcinogenic agent, which produces the primary metabolic activation resulting in initiation of liver carcinogenesis and phenobarbitone is used as promoting agent is normally used to induce liver cancer in several animal models.[4] The present study was aimed to evaluate the anti-cancer Activity in Vasaguluchyadi Kashayam on DEN and phenobarbitone Induced hepatocarcinogenesis in rats and detection and estimation of constituents responsible for therapeutic efficacy.

Figure 1: Vasaguluchyadi Kashayam.

MATERIALS AND METHODS

Collection of Formulation

Vasaguluchyadi kashayam was procured from Kottakkal Arya Vaidya Sala PVT. LTD Coimbatore, Tamilnadu, India.

Estimation of Total Phenolics and Flavonoids content

Estimation of Total Phenolics content

Folin-Ciocalteau assay method is used to determine the total phenolic content.[5,6] Standard solution of Gallic acid (10, 20, 40, 60, 80 and 100 µg/ml) or to an aliquot 100 µl of formulation added 50 µl of Folin-Ciocalteau reagent followed by 860 µl of distilled water and the mixture is incubated for 5 mins at room temperature. 890 µl of distilled water and 100 µl of 20% sodium carbonate were added to make the final solution to 2 ml. It was incubated for 30 mins in dark to complete the reaction. The absorbance of the mixture was measured at 725 nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. The total phenolic content was found out from the calibration curve of Gallic acid. And it was expressed as milligrams of Gallic acid equivalents (GAE) per gram.

Estimation of Total Flavonoids content

Aluminium chloride colorimetric method is used to determine the total flavonoids content.[7] To an aliquot of 100 µl of formulation or standard solutions ethanol was added separately to make up the solution upto 2ml. Then the mixture was treated with 0.1 ml of 1M potassium acetate, 0.1 ml of 10% aluminium chloride and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30 minutes. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1ml potassium acetate, 2.8ml distilled water and 0.1ml of aluminium chloride serve as blank solution.

***In vitro* Antioxidant studies**

DPPH radical scavenging assay

The antioxidant activity of the formulation was measured by Blois method. 0.3mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample (10, 20, 40, 60, 80 and 100µg/ml) and the reference compound (5, 10, 15, 20, 25 and 30µg/ml), shaken vigorously, left to stand in the dark at room temperature for 30min and then absorbance was measured at 517nm against a blank. Reference compound used here was Quercetin. A control reaction was carried out without the test sample.[8] All the tests were performed in triplicate in order to get the mean values. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(AbsControls - AbsSample)]/ (AbsControl)] x 100 where AbsControl is the absorbance of DPPH radical + ethanol; AbsSample is the absorbance of DPPH radical + sample /standard.

ABTS radical cation assay

ABTS radical scavenging activity of the formultion was measured by Rice-Evans method. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation (ABTS*+) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hr before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734nm and equilibrated at 300ºc. Reference compound used here was Quercetin. After addition of 1ml of diluted ABTS + solution to various concentrations of sample (5, 10, 15, 20, 25 and 30µg/ml) and reference compound (0.25, 0.5, 0.75, 1, 1.25 and 1.5µg/ml), the reaction mixture was incubated for 6mins and then absorbance was measured at 734nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition calculated as ABTS radical scavenging activity (%) = [(AbsControl - AbsSample)]/(AbsControl)] x 100

Ferric reducing anti-oxidant power assay (FRAP)

The principle involved in the assay is the reduction of the ferric complex of $Fe(TPTZ)^{3+}$ i.e: tripyridyltriazine (a ferroin analogue) to $Fe(TPTZ)^{2+}$ complex (intensely blue in color) in the presence of anti-oxidant in an acidic pH. There will be an increase in absorbance value at

593nm. The reagent was prepared by mixing 15.15ml of 0.2M acetate buffer (pH 3.6), 1.5ml of 20mMol TPTZ, 1.5ml of 20mMol ferric chloride and 1.9ml of distilled water. The sample /standard compounds were taken in different concentrations of (10, 20, 40, 60, 80 and 100µg/ml) and mixed with 0.9ml of the reagent. Absorbance of the mixture was measured spectrophotometrically at 595nm after 30 minutes of incubation. Quercetin was used as standard. The experiment was done in duplicate and parallel blank was also prepared excluding the sample/standard compound.

Phosphomolybdenum reduction assay

Samples were dissolved in methanol to obtain a concentration of 500 µg/ml. 3 ml of formulation was placed in a test tube, 0.3 ml of reagent solution (0.6 M Sulphuric Acid, 28mM Sodium Phosphate, 4mM Ammonium molybdate) was then added and the resulting mixture was incubated at 950ºC for 90 minutes. After the mixture was cooled to room temperature, the absorbance of the each solution was measured using UV-Visible spectrophotometer at 695nm against blank. The experiment was performed in triplicate.[9] A calibration curve was constructed, using ascorbic acid (100-500 µg/ml) as standard and total antioxidant activity of extract (µg/ml) expressed as ascorbic acid equivalents.

Pharmacological Study

Experimental Animals

Sprague Dawley male rats (150-200gm) were housed under controlled conditions of temperature (20-25^{0}c) and photoperiod 12-h light/dark cycle. All animal procedures were performed after approval from the animal ethical committee and accordance with the recommendations for the proper care and use of laboratory animals.[10,13]

Screening of anti-cancer activity of Vasaguluchyadi kashayam

Thirty male Sprague Dawley rats were used for this study. The animals were divided in to five groups, six animals in each group. DEN 200mg/kg was injected intraperitonealy by dissolving in normal saline and phenobarbitone (0.05%) was given orally through drinking water for the induction of hepatocarcinoma in all the groups except Group I from day 0. After two weeks, the treatment was started such that the normal group (group 1) was treated with saline, Group II was treated with DEN (200 mg/kg *i.p*) and phenobarbitone(0.05% p.o. in drinking water), Group III received standard treatment (5-flurouracil 20 mg/kg *i.p*). Group IV was treated with formulation low dose (500 mg/kg *p.o*) and Group V with formulation high dose (750 mg/kg *p.o*) respectively.

Estimation of Haematological parameters

Blood Collection

After the end of the treatment period (90 days) the animals were anesthetized with anesthetic ether and blood was collected from the retro-orbital vein of rats to analyze hematological parameters such as RBC, WBC and Hb.

Separation of serum

For the estimation of the biochemical parameters such as Serum glutamate oxalo acetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), ALP, Serum creatinine, urea, total bilirubin and total protein, the serum was separated from the blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum was collected and used for the estimation of biochemical parameters.

Evaluation of anti-oxidant parameters

A 10% homogenate of liver tissue was used to analysis of enzymatic antioxidant activity like superoxide dismutase (SOD) Catalase (CAT), and non enzymatic anti oxidant activity reduced glutathione activity (GSH), and lipid peroxidation (LPO) were also estimated.[14,15]

Histopatological studies

The liver from all groups were removed rapidly, and thoroughly rinsed with ice-cold saline. After 24h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide and stained with hematoxylin-eosin for histological assessment of the tissue. Sections of liver were examined by light microscope at 10 and 40X magnification.[16]

Statistical Analysis

The data's of all the parameters were analyzed using the Graph pad 5.0 software. Analysis of Variance (ANOVA); one way ANOVA followed by Tukey multiple comparison test was performed. The values were expressed as Mean ± SEM.

RESULT AND DISCUSSION

Estimation of total phenols and flavonoids content

Total phenols and flavonoids were examined by UV spectrophotometric method. Total phenolic and flavonoids content of the vasaguluchyadi kashayam were found to be

52.06mg/g calculated as Gallic acid equivalent and 67.84 mg/g calculated as quercetin equivalent.

***In vitro* Antioxidant Activity**

The ayurvedic formulation VASAGULUCHYADI KASHAYAM was compared with standard markers to evaluate their antioxidant capacity using DPPH photometric assay, ABTS radical scavenging activity, Ferric reducing ability (FRAP) and Phosphomolybdenum Assay.

Table 1: Indicates the IC_{50} values of standard and the AF.

Assay	IC_{50} value	
	Standard	AF
ABTS(Quercetin)	0.2225	5.962
DPPH(Quercetin)	26.56	42.80

FRAP assay is the reduction of ferric complex(intensely blue in colour) in the presence of anti-oxidant in an acidic pH. There will be an increase in absorbance value Quercetin is used as standard. Absorbance was measured spectrophotometrically at 595nm.

The Phosphomolybdenum reduction assay was based on the reduction of Mo(VI) to Mo(V) in presence of anti-oxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acidic pH and at higher temperature. Phosphomolybdenum assay is used to investigate the total anti-oxidant capacity of Vasaguluchyadi kashayam was found to be 71mg/gm of formulation calculated as Quercetin equivalent.

Pharmacological Activity

Haematological parameters

The results of haematological investigation of disease control group showed a significant reduction of RBC, WBC and Hb levels. The standard and formulation treated groups showed a significant increase of RBC, WBC and Hb levels. (Table 2).

Effect of Body weight and liver weight analysis in rats

The results revealed that the body weights of rats were significantly decreased in DEN treated group. However, formulation treatment groups showed significant increased in body weight when compared to DEN control group. Standard drug also significantly increased the body weight than the DEN treated animals but no significant difference was observed between formulation treated animals and standard treated animals. (Table 3) Liver weight of rats were

significantly increased in DEN control group. However, formulation treated groups were showed significantly increased in liver weight whereas, Standard drug showed significant reduction in relative liver weight compared to DEN control group.(Table 5).

Serum biochemical parameters

In DEN treated group, the level of SGOT, ALP, SGPT, urea, total bilirubin, total protein and creatinine were significantly elevated. The formulation treated groups were able to reverse all the elevated serum biochemical parameter near to normal and the results were comparable to that of standard treated group. (Table 4).

***Invivo* antioxidant studies**

DEN induced control group increased the level of LPO and decreased the level of CAT, SOD and GSH levels in liver homogenate. The formulation preventive groups manifested remarkable increase in SOD, CAT, GSH levels and decreased the LPO level were compared to disease control group. (Table 6).

Table 2: Effect of AF on RBC WBC and Hb.

Groups	Normal	Control	Standard	Formulation low dose	Formulation high dose
RBC ($1x\ 10^6$ cells/mm^3)	9.92±0.15	5.99±0.12***	8.48±0.16***	$7.27±0.16^{***}$	$6.89±0.21^{**}$
WBC ($1x\ 10^3$ cells/mm^3)	5.88±0.05	4.60±0.08***	5.71±0.08***	4.97±0.08*	$4.85±0.07^{ns}$
Hb(gm/dl)	16.70±0.19	13.01±0.04***	15.85±0.09***	14.85±0.20***	15.32±0.17***

Values are expressed as mean ± SEM; (n=6); (One way ANOVA, Tukey, multiple comparison test). Normal vs control group. Standard and Test groups vs. Control. (*** P<0.0001, ** P< 0.001, * P< 0.05, ns-non significant).

Table 3: Effect of AF on the Body weight of the experimental rats.

Weight in gram	Normal	Control	Standard	Formulation Low dose	Formulation High dose
Initial body weight	163.7±2.2	177.7±3.6*	135.8±3.8***	134±2.3***	147±3.5***
Final body weight	179.3±3.2	154±3.483***	162.5±3.274*	$163±2.7^{ns}$	170.5±4.0**

Values are expressed as mean ± SEM; (n=6); (One way ANOVA, Tukey, multiple comparison test). Normal vs control group. Standard and Test groups vs. Control. (*** P<0.0001, ** P< 0.001, * P< 0.05, ns-non significant).

Table 4: Effect of the AF on the Serum biochemical Parameters.

Groups	Normal	Control	Standard	Formulation Low dose	Formulation High dose
SGOT(U/L)	46.45±1.906	92.67±2.287***	77.29±1.852***	81.33±1.961**	76.23±2.061***
SGPT(U/L)	39.05±0.9969	85.22±1.540***	60.53±2.312***	72.43±2.667***	65.60±1.423***
ALP(U/L)	85.67±1.868	145±3.663***	93.82±2.649***	112.6±3.256***	105.2±3.247***
UREA(mg/dl)	29.32±0.831	66.65±3.120***	54.87±2.587*	58.82±2.691^{ns}	52.23±2.766**
CREATININE (mg/dl)	0.63±0.018	1.68±0.108***	0.68±0.018***	0.72±0.018***	0.78±0.016***
TOTALPROTEIN (g/ml)	4.38±0.198	7.40±0.390***	5.10±0.273***	4.60±0.283***	6.01±0.238*
TOTAL BILLIRUBIN (mg/dl)	0.71±0.048	1.68±0.072***	0.93±0.037***	0.78±0.053***	1.287±0.048***

Values are expressed as mean ± SEM; (n=6); (One way ANOVA, Tukey, multiple comparison test). Normal vs control group. Standard and Test groups vs. Control. (*** P<0.0001, ** P< 0.001, * P< 0.05, ns-non significant).

Table 5: Effect of the AF on the Liver weight of the experimental rats.

Groups	Normal	Control	Standard	Formulation low dose	Formulation high dose
Liver weight (gm)	8.38±0.14	9.16±0.10***	8.02±0.09***	7.64±0.16***	7.00±0.02***

Values are expressed as mean ± SEM; (n=6); (One way ANOVA, Tukey, multiple comparison test). Normal vs control group. Standard and Test groups vs. Control. (*** P<0.0001, ** P< 0.001, * P< 0.05, ns-non significant).

Table 6: Estimation of enzymatic and non enzymatic activity.

Groups	Normal	Control	Standard	Formulation low dose	Formulation high dose
SOD (U/min/mg/protein)	7.930±0.09	3.068±0.22***	6.982±0.14***	6.863±0.15***	6.455±0.15***
CAT (µmol of H_2O_2 consumed/ min/mg protein	79.88±0.96	50.00±2.38***	73.41±1.07***	58.74±0.95**	61.89±0.93***
LPO (nmol MDA / mg protein)	0.64±0.05	5.71±0.17***	1.74±0.22***	2.01±0.21***	2.71±0.23***
GSH (mmoles/mg tissue protein)	9.74±0.09	4.22±0.17***	8.93±0.08***	7.31±0.13***	6.94±0.13***

Values are expressed as mean ± SEM; (n=6); (One way ANOVA, Tukey, multiple comparison test). Normal vs control group. Standard and Test groups vs. Control. (*** P<0.0001, ** P< 0.001, * P< 0.05, ns-non significant).

Estimation of Histopathology

Normal Control

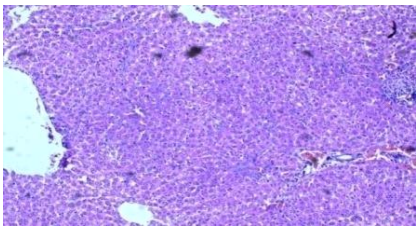

Figure 2: Normal group showing the no evidence of inflammation and liver fibrosis.

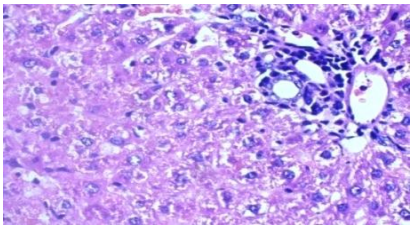

Figure 3: DEN induced control group showing portal tract portal inflammation are present. Parenchymal necrosis (Focal lytic necrosis) was also evident.

Standard Formulation Low Dose

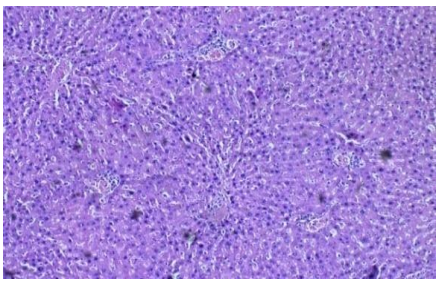

Figure 4: Standard drug treated group showing mild portal inflammation No evidence of parenchymal inflammation/Necrosis.

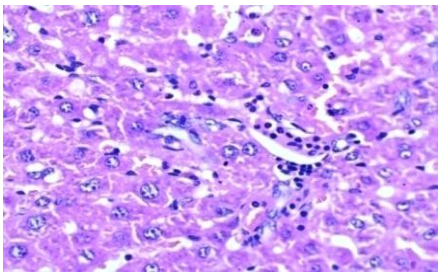

Figure 5: Formulation treated low dose group showing portal tract- inflammation present. Hepatic parenchyma – Mild necrosis.

Formulation High Dose

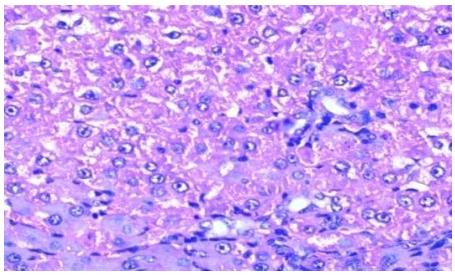

Figure 6: Formulation high dose treated group showing portal inflammation with interface hepatitis. Parenchymal marked inflammation.

DISSCUSION

This study demonstrate a potential role of Vasaguluchyadi Kashayam in limiting pre neoplastic changes in DEN and Phenobarbitone-initiated experimental hepatocarcinogenesis in rats. Total phenolic and flavonoids content of the vasaguluchyadi kashayam were found to be 52.06mg/g calculated as Gallic acid equivalent and 67.84 mg/g calculated as quercetin equivalent.[17,18] In the DPPH, ABTS, phosphomolybdenum and FRAP radical scavenging assay, formulation has displayed the highest antiradical activity in both assays and was also comparable with the standard qucertein. Increase in liver weight of rats were observed in the disease control group and the significant reduction of liver weight of rats were observed in both standard and formulation treated groups. Alterations were seen in the hematological parameters in HCC. The disease control group has shown a significant reduction in RBC, WBC and Hb levels. The standard and formulation treated group has shown a significant increase of RBC, WBC and Hb levels. Serum biochemical parameters showed an altered pattern in the cancerous condition. The increase in the level of SGOT, SGPT, and ALP in blood indicated that DEN may prompt hepatic dysfunction. The enzymes directly associated with the conversion of amino acids to keto acids are SGOT and SGPT, and are proved to be increased in the HCC condition. Serum transaminases, alkaline phosphatases, total bilirubin, creatinine, urea and a tumor marker enzyme alpha feto protein were evaluated. Formulation treated groups showed effective and moderate activity in normalizing the parameters. Total Protein present in the liver tissue of disease control group was reduced due to the cancerous condition. But in the formulation preventive groups there were a significant increase in the protein levels compared to disease control group. Formulation treated groups also exhibited significant increase in the protein content, bringing back it to the normal. The impaired liver functions may be due to oxidative stress which leads to disturbances in the protective physiological moieties that further leads to lipid peroxidation thus eventually causes damage

to the macromolecules in vital biomembranes. Alteration in the activity of enzymatic as well as non-enzymatic components such as SOD, CAT, LPO and GSH levels in the disease control group, while it's note worthy that the Formulation treated group manifested remarkable increase in their level (SOD, CAT, GSH) which was extremely significant even more than that observed with standard group. The results obtained from the current study demonstrate that the Formulation offers a significant protection against hepatocarcinoma and it was very clear that the formulation was able to prevent malignancy. It was clear that there exists an antioxidants, When the Formulation groups received showed higher shielding in DEN and Phenobarbitone induced HCC rat model. When the formulation was given for prevention and treatment of cancer, the formulation has manifested propitious results which may be on account of the additive activity of the constituents present in the formulation.

CONCLUSION

The present study it can be concluded that the Vasaguluchyadi Kashayam has the potential to prevent HCC as well as it can reduce the complications of the same. It seems promising that these data obtained from the study can be further validated in the future studies which eventually can be developed as a formulation that offers a high degree of protection from HCC.

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ABBREVIATION USED

Abbreviation Full name/ Meaning

AF Ayurvedic Formulation

HCC Hepatocellular Carinoma

DEN Diethylnitrosamine

ROS Reactive Oxygen Species

GSH Reduced Glutathione

SOD Superoxide Dimutase

CAT Catalase

LPO Lipid Peroxidation

SGOT Serum Glutamate Oxaloacetate Transminase

SGPT Serum Glutamate Pyruvate Transaminase

p.o Per Oral

i.p Intra peritoneal

µl Micro Litre

ml Milli Litre

ANOVA Anaysis of Variance

SEM Standard Error Mean

SD Standard Deviation

ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid

DPPH 2,2-diphenyl-1-picrylhydrazyl

REFERENCES

1. Chuang SC, La Vecchia C, Boffetta P. Liver cancer: Descriptive epidemiology and risk factors other than HBV and HCV infection .Cancer lett., 2009; 286(1): 9-14.
2. Gurfateh singh, Nancy dhadwal, Harikumar sl Experimen tal models for hepatotoxicity. Asian journal of pharmaceutical and clinical research, 2015; 8(2): 70-74.
3. Dr.Nidhi Mishra et al., Enicostemma littorale blume review article. Journal of medicinal plant studies, 2017; 5(1): 78-82.
4. K. Balamurugan et al., Evaluation of Luteolin in the Preventionof N-nitrosodiethylamine-induced Hepatocellular Carcinoma Using Animal Model System Ind J Clin Biochem, 2012; 27(2): 1-2.
5. Leelaprakash G, Mohan Dass S. Antimicrobial activity and phytochemical screening of methanol extract of Enicostemma axillare. Int J Pharm Pharm Sci., 2012; 4(1): 342–348.
6. Ragazzi, E,. Veronese G. Quantitative analysis of phenolics compounds after thin–layer chromatographic separation. J. Chromatogr, 1973; 77: 369.375.
7. Zou YP, Lu YH, & Wei DZ. Antioxidant activity of a flavonoid rich extract of Hypericum perforatum L. in vitro. J Agric Food Chem., 2004; 52: 5032-5039.
8. Akhtar H. Antioxidant potential of dried Enicostemma littorale. Pak Bio Sci., 2011; 14(20): 956.
9. Krishnaveni A, Mohandass S. Antioxidant activity of Enicostemma axillare (invitro studies). Journal of pharmacy research, 2012; 5(8): 3954-3956.
10. Verna L, Whysner J and Williams GM: N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol Ther., 1996; 71(1-2): 57-81.

11. Bhatt NM, Barua S, Gupta S. Protective effect of Enicostemma littorale Blume on rat model of diabetic neuropathy. Am J Infect Dis., 2009; 5(2): 99–105.
12. Gupta S, Dadheech N, Singh A, Soni S, Bhonde RR. Enicostemma littorale: A new therapeutic target for islet of neogenesis. Int J Int Bio., 2010; 9(1): 50-53.
13. Rajasekaran A, Arivukkarasu R, Murugesh S. Hepatoprotective effect of Adenema hyssopifolium G.Don (Gentianaceae) in carbon tetrachloride-induced hepatotoxicity in rats. Trop J Pharm Res., 2010; 9(2): 157–163.
14. Thirumalai T, Therasa VS, Elumalai EK, David E. Hypolipidemic and antioxidant effect of Enicostemma littorale Blume. Asian Pac J Trop Biomed., 2011; 1: 381–385.
15. Sathiskumar R, Lakshmi PTV, Annamalai A. Comparative analyses of non enzymatic and enzymatic antioxidants of Enicostemma littorale Blume. Int J Pharma Bio Sci., 2010; 1(2): 1– 16.
16. Janani P, Sivakumari K, Geetha A, Ravisankar B and Parthasarathy C: Chemopreventive effect of bacoside a on n-nitrosodiethylamine-induced hepatocarcinogenesis in rats. J Cancer Res Clin Oncol, 2010; 136: 759-770.
17. He RY and Nie RL. Studies on bitter principles from Swertia mileensis. Acta Botanica yunnanica, 1980; 2: 480-82.
18. Zhou J. some bioactive substances from plants of west china. Pure and Applied Chemistry, 1989; 61: 457-60.