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Evaluation of Antitumor and Antioxidant activity of Flavonoid fraction of *Terminalia Catappa* against Ehrlich Ascites Carcinoma in Mice

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Abstract

Antitumor activity of flavonoid fraction of *Terminalia catappa* (TcFf) was evaluated against Ehrlich Ascites Carcinoma (EAC) in mice. After 24 hour tumor inoculation the extract was administered for the period of 15,th30,th45th and 60 days. After administration of the last dose followed by 18 h fasting, mice were sacrificed for observation of antitumor activity for each treatment period. The activity of enzymic antioxidants such as Catalase (CAT), Superoxide dismutase (SOD) and glutathione peroxidase (GPX) and the levels of non enzymic antioxidants such as Vitamin A, Vitamin E and Reduced glutathione (GSH) and lipid peroxide (MDA) in the liver homogenate of control and experimental mice were determined. The activities of enzymic antioxidants and the levels of non enzymic antioxidants were decreased in ELA control mice and MDA level was increased. Administration of TcFf significantly altered the antioxidants level and MDA to normal level. The result suggests that flavonod fraction of *Terminala catappa* exhibited antitumor effect by modulating lipid peroxidation and augmenting antioxidant defense system in EAC bearing mice.

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Terminalia catappa, ELA, antioxidants, lipid peroxidation

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INTRODUCTION

Cancer is the second leading cause of death worldwide next to cardiovascular diseases and can be

treated with surgery, radiation, chemotherapy, hormone therapy [1] and biological therapy [2] .Emerging evidence suggests that a number of plants are known to be the source of useful drugs in modern medicine and have been accepted currently as one of the main source of cancer chemoprevent drug discovery and development [3,4] due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [5,6]. Intraperitoneally propagated ELA tumor cells in Swiss albino mice are an important experimental approach to study the biology of human cancer. The methanolic extract of aerial parts of *Momordica cymbalaria* hook F, crude extract and diethylether fractions of *Alangium salvifolium* flower and ethanolic extract of *Tragia Plukenetii* R.Smith were found retard the tumor development and increased the life span of ELA tumor bearing mice [7-9].

Terminalia catappa is (family - combretaceae) also known as badam widely grown in tropical regions of the world as an ornamental tree. It is found in the warmer parts of India. It is also known as Indian Almond, Malabar Almond, and Tropical Almond. The aqueous and cold extracts of leaves of the *Terminalia catappa* have been reported to be antioxidant, hepatoprotective, and anti-diabetic [10].We have already reported that *Terminalia catappa* leaf protein has antioxidant activity against ELA implanted Swiss albino mice [11]. In the present study was designed to determine the antioxidant and antitumor activity of flavonoid fraction of *Terminalia catappa* against ELA implanted Swiss albino mice.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Terminalia catappa* was collected in area free of pesticides and other contaminants from the area surrounding of Coimbatore,Tamilnadu.The collected leaves were washed thoroughly and blotted dry with filter paper and used for the flavonoid fraction preparation**.**

Preparation of flavonoid fraction

In the preliminary screening, the direct ethyl acetate extract of *Terminalia catappa* with powdered magnesium + conc.HCl developed an orange to magenta color indicated the presence of flavonoid showed a characteristic color reaction in Shinoda test. The color is due to the reductive conversion of the flavone into the corresponding anthocyanin pigment [12].Knowing the presence of flavonoid in ethyl acetate extract, the extraction was undertaken with 20 g of powdered plant material and 200ml. of light petroleum ether (b.p. 40^{o} – 60^{o} C) in a Soxhlet apparatus for 18 hours to remove the chlorophyll, non flavonoid components and lipid de waxing[13] The treated material was dried and extracted with ethyl acetate using Soxhlet apparatus.[14]This fraction is referred as TcFf.

Animals.

Seven to eight weeks old Swiss albino male mice weighing about 25-30 g were brought from small animals breeding station, Thrissur, Kerala. The animals were acclimatized for 60 days under standard laboratory conditions and fed with standard diet with water ad libitum.

Propagation of ELA cell lines

Ehrlich's Lymphoma Ascites (ELA) tumor cell lines were procured from Amala Cancer Research Centre, Thrissur, Kerala.The mice were acclimatized for two weeks and cells were propagated by intraperitoneal transplantation of $1x10^{6}$ cells in 100µl of PBS. After 15 days, the cells were drawn from the intraperitoneal cavity and used for the *in vitro* cytotoxic studies by trypan blue exclusion method.[15] .In vitro cytotoxic studies were carried out to find out the 50% effective dose (ED_{50}) of *Terminalia catappa* flavonoid fraction which was 75µg/100µl determined by trypan blue exclusion method. The fraction which showed minimum ED_{50} was selected for the in vivo studies. All animal experiments were carried out according to the guidelines prescribed by Animal

Welfare Board and with the approval of Animal Ethic Committee (Register no: 623/02/b/CPCSEA).

Grouping of animals and Treatment Schedule

The animals were divided in to seven groups with 6 mice in each for each treatment period.The grouping and treatment of animal as follows.

Group 1 received (i.p) 0.1 ml of PBS every day and served as a vehicle control for the experimental group 6 & 7

Group 2 received (i.p) 0.1 ml of paraffin oil, every day and served as a vehicle control for the standard antioxidant silymarin group

Group 3 received (i.p) 0.1 ml of Dimethyl sulphoxide (DMSO) every day and served as a vehicle control for the experimental groups 5 & 6.

Group 4 received (i.p) 25mg standard antioxidant silymarin in 100 µl of paraffin oil / kg body weight.

Group 5 received (i.p) 75µg (ED_{50}) of TcFf in 100µl of DMSO.

Group 6 received 1x10^6 ELA tumor cells and 75µg of TcFf (i.p) on the same day and TcFf administration was continued for 60 days (TcFf + ELA)

Group 7 received 1x10^6 ELA tumor cells (i.p) that served as ELA control

The study was continued for the period of 15 days, 30 days, 45 days and 60 days. At the end of the each treatment the mice were sacrificed after an overnight fasting. The liver was dissected, blotted of blood, washed with PBS at pH 7.2 and homogenate was prepared using PBS and used for the determination of CAT, [16] SOD [17], GPx [18] and the non enzymic antioxidants such as vitamin A [19] vitamin E [20] and GSH [21]. A part of the liver homogenates were prepared using Tris HCl for the assessment of the rate of lipid peroxidation.[22]

STATISTICAL ANALYSIS

The data presented here are means ± SD of 6 mice in each group. The biochemical estimations using mice for 15 days treatment period alone including ELA were subjected to one-way ANOVA and the results of 15 days to 60 days treatment periods excluding ELA treated group were analysed using two way ANOVA using SigmaStat statistical package to test the level of statistical significance at $P<0.05$

RESULT AND DISCUSSION

In the human body the free radicals are continuously produced due to the oxygen utilization by the cells of the body. This generates a series of reactive oxygen species (ROS) like super oxide anion (O_2^{-}) and hydroxyl (HO·) radicals and non-free radical species such as H_2O_2, singled oxygen and nitric oxide (NO).Reactive oxygen species and reactive nitrogen species are associated with many pathological conditions such as atherosclerosis, ischemia, and reperfusion injury of many tissues, central nervous system injury, gastritis and cancer [23,24]. Phytocompounds like flavonoids and phenolic acids, commonly found in plants [25] have been reported to have multiple biological and pharmacological activities including antioxidative ,cytotoxic, anticancer [26-28]antimicrobial, antiviral [29]and anti–inflammatory activities [30,31].

Antioxidants cause protective effect by neutralizing free radicals, which are toxic byproducts of natural cell metabolism. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases[32].

The liver occupies a vital role in the main functions of the organism. It is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogeneity. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) formation and scavenging by antioxidants. Excess generation of

ROS can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis[33]. The ELA tumor bearing mice life span was found to be 15-25days with the average life span of 19 days.

Effect on enzymic antioxidants

Table 1 shows the activities of enzymic antioxidants in the liver of control and experimental animals. SOD is a ubiquitous chain-breaking antioxidant found in all aerobic organisms. It is a metalloprotein widely distributed in all cells and plays an important protective role against oxidative damage induced by reactive oxygen species. SOD converts superoxide ion(O2-) to hydrogen peroxide (H_2O_2) and the hydrogen peroxide thus formed is degraded by CAT and GPx. CAT is present in all major body organs of animals and humans and is especially concentrated in the liver.[34,35].

Table 1: Activities of enzymic antioxidants in the liver of control and experimental Swiss albino mice

Treatment Groups	CATALASE (U / mg protein)[a]				SUPEROXIDE DISMUTASE (U / mg protein)[a]				GLUTATHIONE PEROXIDASE (U / mg protein)[c]			
	15 days	30 days	45 days	60 days	15 days	30 days	45 days	60 days	15 days	30 days	45 days	60 days
PBS	6.41 ± 0.102	6.451 ± 0.114	6.50 ± 0.151	6.46 ± 0.123	2.12 ± 0.077	2.19 ± 0.059	2.211 ± 0.037	2.271 ± 0.050	0.276 ± 0.021	0.316 ± 0.017	0.310 ± 0.032	0.306 ± 0.021
Paraffin oil	6.16± 0.148	6.188 ± 0.11	6.27 ± 0.04	6.29 ± 0.042	2.025 ± 0.114	2.155 ± 0.034	2.215 ± 0.047	2.20 ± 0.060	0.26 ± 0.016	0.281 ± 0.019	0.291 ± 0.019	0.291 ± 0.0194
DMSO	6.31 ± 0.17	6.25 ± 0.1581	6.27 ± 0.154	6.236 ± 0.053	2.16 ± 0.149	2.201 ± 0.136	2.22 ± 0.129	2.22 ± 0.10	0.22 ± 0.015	0.23 ± 0.0178	0.236 ± 0.019	0.241 ± 0.026
Silymarin	6.65 ± 0.184	7.05 ± 0.24	7.316 ± 0.347	8.133 ± 0.040	2.30 ± 0.18	2.78 ± 0.09	3.18 ± 0.27	3.26 ± 0.27	0.34 ± 0.041	0.81 ± 0.05	1.08 ± 0.22	1.19 ± 0.19
TcFf	7.58 ± 0.20	7.95 ± 0.09	8.07 ± 0.17	8.69 ± 0.31	2.40 ± 0.14	2.85 ± 0.07	3.30 ± 0.30	3.54 ± 0.16	0.461 ± 0.061	0.87 ± 0.06	1.21 ± 0.16	1.38 ± 0.14
ELA+TcFf	5.25 ± 0.24	5.92 ± 0.13	6.84 ± 0.42	7.23 ± 0.36	1.89 ± 0.08	2.006 ± 0.127	2.89 ± 0.22	3.08 ± 0.27	0.403 ± 0.031	0.738 ± 0.077	0.99 ± 0.156	1.013 ± 0.135
ELA	3.47 ± 0.24	–	–	–	0.85 ± 0.06	–	–	–	0.055 ± 0.01	–	–	–

The values are the mean ± SD of six animals P<0.05

a Amount of enzyme required to decrease the absorbance by 0.05 Units at 240 nm.

b Amount of enzyme that gives 50 per cent inhibition of the extent of NBT reduction.

c Microgram of GSH utilized per min per milligram protein.

In the present study ELA tumor induced mice showed a significant decrease in CAT, SOD and GPx activities on 15 days of treatment period when compared to all the controls and all the treatments. Catalase, SOD and GPX activities were found to be significantly increased in TcFf administration to ELA tumor induced mice on all treatment periods when compared to 15 days treatment period. The decreased activities of enzymic antioxidants in ELA induced liver reduces the protection against free radicals. These enzymic antioxidants activities were found to be increased in mice treated with silymarin and TcFf in all the treatment periods when compared to their respective controls. TcFf administration showed significant increase in CAT,SOD and GPx activities than that of silymarin administration on 30,45 and 60 days when compared to 15 days treatment period which confirms the antioxidative and antitumorigenic role of TcFf.

Effect on non enzymic antioxidants

The levels of non-enzymic antioxidants such as vitamin A and vitamin E and Reduced glutathione(GSH) in the liver of Swiss albino mice on different treatment periods are shown in the Table 2.

Table 2: Activities of non-enzymic antioxidants in the liver of control and experimental Swiss albino mice

Treatment Groups	VITAMIN A (µg/g tissue)				VITAMIN E (µg/g tissue)				REDUCED GLUTATHIONE (nmoles /g tissue)			
	15 days	30 days	45 days	60 days	15 days	30 days	45 days	60 days	15 days	30 days	45 days	60 days
PBS	0.671 ± 0.30	0.72 ± 0.029	0.728 ± 0.024	0.725 ± 0.024	2.18 ± 0.12	2.25 ± 0.07	2.27 ± 0.068	2.225 ± 0.087	10.11 ± 0.153	10.29 ± 0.086	10.28 ± 0.082	10.30 ± 0.076
Paraffin oil	0.73 ± 0.042	0.74 ± 0.04	0.748 ± 0.034	0.755 ± 0.025	2.23 ± 0.096	2.30 ± 0.072	2.29 ± 0.081	2.303 ± 0.09	10.17 ± 0.21	10.35 ± 0.075	10.36 ± 0.067	10.40 ± 0.026
DMSO	0.70 ± 0.08	0.713± 0.019	0.705 ± 0.0266	0.698 ± 0.023	2.24 ± 0.152	2.365 ± 0.174	2.265 ± 0.148	2.258 ± 0.160	10.25 ± 0.45	10.28 ± 0.627	10.196 ± 0.577	10.278 ± 0.47
Silymarin	0.806 ± 0.116	1.30 ± 0.03	1.51 ± 0.16	2.01 ± 0.30	3.31 ± 0.316	3.678 ± 0.108	3.91 ± 0.281	4.20 ± 0.244	11.54 ± 0.26	11.971 ± 0.20	12.15 ± 0.316	12.578 ± 0.224
TcFf	0.925 ± 0.072	1.62 ± 0.15	1.86 ± 0.213	2.21 ± 0.21	3.49 ± 0.233	3.77 ± 0.068	4.225 ± 0.26	4.44 ± 0.237	11.613 ± 0.249	12.10 ± 0.33	12.55 ± 0.127	12.913 ± 0.229
ELA+TcFf	0.90 ± 0.12	1.29 ± 0.03	1.54 ± 0.249	2.04 ± 0.27	3.11 ± 0.10	3.69 ± 0.62	3.94 ± 0.306	4.05 ± 0.25	9.90 ± 0.652	10.361 ± 0.317	10.84 ± 0.344	12.31 ± 0.528
ELA	0.63 ± 0.07				2.04 ± 0.272				8.76 ± 0.20			

The values are the mean ± SD of six animals $P<0.05$

GSH is a tripeptide (L-γ-glutamylcysteinylglycine), an antioxidant and a powerful nucleophile, critical for cellular protection such as detoxification from reactive oxygen species, conjugation and excretion of toxic molecules, and control of the inflammatory cytokine cascade[36] .Depletion of GSH in tissues leads to impairment of the cellular defence against reactive oxygen species, and may result in peroxidative injury. Vitamin A is a fat-soluble vitamin, which is essential for growth maintenance and differentiation of epithelial cells. Vitamin A breaks the chain of lipid per oxidation to cell membrane and prevents the formation of lipid peroxide [37]. Vitamin E is a major lipid phase antioxidant that protects against oxidative lipid damage. It scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting PUFA present in cell membrane.In the present study ELA tumor induced mice showed a significant decrease in Vitamin A and Vitamin E and GSH levels on 15 days of treatment period when compared to all the controls and all the treatments. Vitamin A and Vitamin E and GSH levels were found to be significantly increased in TcFf administration to ELA tumor induced mice on all treatment periods when compared to 15 days treatment period. Vitamin A and Vitamin E and GSH levels were found to be increased in mice treated with silymarin and TcFf in all the treatment periods when compared to their respective controls. TcFf administration showed significant increase in the levels of these non enzymic antioxidants than that of silymarin administration on 30,45 and 60 days when compared to 15 days treatment period which confirms the antioxidative and antitumorigenic role of TcFf.

The effect of TcFf, silymarin and ELA on lipid peroxidation in the liver of Swiss albino mice are given in Table 3.

Table 3: levels of lipid peroxide(nmoles of MDA/mg protein in liver of controls and experimental mice

Treatment Groups	TREATMENT PERIOD IN DAYS			
	15 days	30 days	45 days	60 days
PBS	0.214±0.0153	0.216±0.01	0.204±0.010	0.208±0.01
Paraffin oil	0.207±0.006	0.199±0.0089	0.205±0.01	0.204±0.009
DMSO	0.2038±0.005	0.202±0.006	0.203±0.007	0.205±0.006
Silymarin	0.173 ± 0.019	0.156 ± 0.021	0.13 ± 0.011	0.118±0.024
TcFf	0.191 ± 0.020	0.148 ± 0.019	0.141 ± 0.014	0.136± 0.016
TcFf+ELA	0.198 ± 0.017	0.194 ± 0.016	0.178 ±0.017	0.171 ±0.014
ELA	0.253±0.0098	-	-	-

The values are the mean ± SD of six animals $P<0.05$

MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid .[38] Therefore, the hepatic content of MDA is used as an indicator of liver tissue damage involving a series of chain reactions.[39] The mice transplanted with ELA tumor cells showed a significant increase in MDA level when compared to all the controls and experimental groups in 15 days treatment period. Coadministration of TcFf to ELA tumor induced mice showed significant decreased levels of MDA in all the treatment period.The level of MDA in liver was found to be significantly decreased in all the treatment periods by the administration of TcFf when compared to DMSO. The level of MDA in the standard antioxidant silymarin treated mice was found to be significantly decreased when compared to paraffin oil treated mice on all treatment periods. Silymarin administration showed more significant decreased levels of MDA in15, 45 and 60 days treatment periods than that of TcFf administered mice. The significant reduced levels of MDA in mice supplemented with the TcFf individually and in ELA tumor induced mice indicated their antilipid peroxidative role

CONCLUSION

On the whole, it can be concluded that flavonoid fraction of *Terminalia catappa* restored the hepatic lipid peroxidation and antioxidant enzymes such as SOD, bearing mice to near normal levels. Therefore, CAT and GPx as well as non enzymic antioxidants vitamin A and E and GSH in tumor further studies should be conducted to determine the active compounds that are responsible for the antitumor effects and the mechanisms of action involved in the antitumorigenic effect.

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