International Journal of Engineering, Science and Mathematics
Vol.7 Issue 4(2), April 2018,
ISSN: 2320-0294 Impact Factor: 6.765
Journal Homepage: http://www.ijmra.us, Email: editorijmie@gmail.com
Double-Blind Peer Reviewed Refereed Open Access International Journal - Included in the International Serial Directories Indexed & Listed at: Ulrich's Periodicals Directory ©, U.S.A., Open J-Gage as well as in Cabell's Directories of Publishing Opportunities, U.S.A

Pharmacological Investigation with Tea Root Extract (*Camellia sinensis*) Depicting its Non-toxicity as Therapeutic Herbal Agent

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Abstract

It has been reported previously that tea root extract and its triterpenoid saponins possess potent antitumour activities and significantly reduced solid tumour in mice. TRE also induced apoptosis in human leukemic cell lines K562, U937 and HL60 cell lines. In the present study we have investigated several other related pharmacological properties to ensure the safe use of TRE as a therapeutic agent in future. The results of the present investigations had depicted that TRE is non-hepatotoxic as it has not altered the SGPT and SGOT level significantly in CCL_4 treated mice and rat.TRE had shown mild and insignificant effect on the inhibition of neuromuscular activities as revealed in the experiment with rat diaphragm. Macrophage activation by TRE can be considered as an important factor related to its anti-cancer activity. And also depict that the extract does not cause immune-suppression. The enhancement in the number of peritoneal cells by tea root extract indicates that TRE administration does not cause death of normal cells.Overall it can be concluded that tea root extract possess promising therapeutic potential due to its insignificant toxicity.

Keywords:

Tea Root extract;
Inflammation;
Anti-oxidant;
Neuro-muscular Effect;
Hepatoprotection;
Macrophage;

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1. Introduction

Roots of medicinal plants are common ingredients of many folk and herbal medicines [1, 2] and root extracts of a number of medicinal plants have been reported to possess pharmacological activity, mainly anti-inflammatory activity [3–7].Many of the potentially therapeutic herbal extracts and the marketised medicines prepapared from them possess

different harmful side effects specially hepatotoxicity, neural disintegration, immune suppression etc. that restrict their use as a good medicine.Since it was previously reported that the Tea Root Extract (TRE) and the triterpenoid saponins isolated from it possess potent anticancer activity, so in the present study we have evaluated different pharmacological effect of Tea Root Extract that should be investigated to establish it as a potent therapeutic agent. The pharmacological properties of tea have been well studied but very little work has been done with the tea root. The present investigation revealed the fact that tea root extract is non-hepatotoxic and does not causes immune suppression. Efforts have also been made to get an insight into the mechanism of action of TRE's positive effects.So the extract has great pharmacological value and could be used safely in different therapies

2. Research Method

Plant Materials: The roots of Camellia sinensis Var assamica (clone TV-1, planted in 1964) were collected and supplied by the Tocklai Experimental Station, Jorhat, India. A voucher specimen is deposited at the Tea Research Association, Kolkata, India.

Extraction and preparation of TRE: The dried tea roots were taken in 50% aqueous methanol and kept for a week at room temperature (20-30°C). The solvent was evaporated in a rotary evaporator and the dry mass was dissolved in
normal saline and used in specific concentration for experiment.

Animals Albino rats (Sprague-Dawley strain) weighing 125-150 g and Balb-C mice weighing 20-25 g bred were used.

Methods

Acute toxicity study: Mice were divided into groups of 10 and TRE was injected i.p. in doses from 10 mg to 200 mg/kg. The LD50 (24 hours) was calculated graphically [8].

Anti-inflammatory activity` Acute model; Carrageenan induced oedema

The rats were divided into three groups (n=6) and the first group served as negative control and received normal saline (0.1ml/100 g i.p). The second group was administered acetyl salicylic acid (100 mg/kg i.p.) as the standard drug. Group 3 received 10 mg/kg i.p. of TRE. Oedema was produced by the method described by Winter et al., 1962 [9]. Carrageenan (0.1 ml/100 g from a 10 mg/ml solution) was injected into the plantar aponeurosis of right hind paw of the rats of all three groups
30 min after drug administration. The left hind paw served as the control. The paw volume was measured after 4 hours using a plethysmometer (UGO BASILE). After measuring the paw volume the rats were killed by cervical dislocation and both the hind paws were cut from the ankle joint and weighed. The weight and volume of the right hind paw was compared with that of the left hind paw [9].

Chronic model: Freund's adjuvant induced poly-arthrities

The method of Newbould, 1963 [10] was followed. 18 male albino rats were divided into three groups of 6 animals in each group. On day 1, 0.1 ml of Freund's complete adjuvant was injected into the right plantar pad of each rat. While rats of group-I received 0.1 ml/100 g of normal saline i.p. daily, those of group-II received 100 mg/kg acetyl salicylic acid i.p. daily and group-III received 10 mg/kg TRE i.p. daily for 21 consecutive days. The paw volume for each group was measured using plethysmometer (UGO BASILE) on day-0

before administration of adjuvant and on day-21 after treatment. Percentage inhibition was thereby calculated. Severity and development of secondary lesions were also compared [10].

Determination of the effect of TRE on Neurotransmission and Skeletomotor activity
Effect on isolated rat diaphragm
Isolated phrenic nerve hemidiaphragm preparation was made using albino rats of either sex, according to the method of Bulbring (1946) [11] and as described by Das et al. (1978) 12].Denervation of the left phrenic nerve was carried out according to the method of Mitchell and Silver (1963) [13] and the preparation was set up according to method of Vedasiromoni and Ganguly (1984) [14].Supramaximal square wave pulses of 0.2 ms duration, at a requency of 0.2 Hz, were used for indirect and direct stimulation.The diaphragm was suspended in a 10 ml organ bath containing Kreb's solution (mM: NaCl 118, KCl 4.7, CaCl2.6H2O 2.5, NaHCO3 25, KH2PO4 1.2, MgSO4.7H2O 1.2 and dextrose 11.1) and continuosly aerated with 95% O2 and 5% CO_2 at a temperature of 32-34°C. In one set of experiments, the concentration of MgCl2 was increased to reduce the twitch height to a steady level of about 50% of the control [15] before administration of TRE. The effect of TRE was examined by using the concentrations of 5, 10 and 15 mg/ml by measuring the twitch response.

Effect on liver, CCl_4 induced hepatotoxicity
Rats were divided into 6 groups of six animals in each group.Three groups received TRE at doses of 5, 10 and 15 mg/kg body weight/day respectively for 3 weeks . Group 4 served as normal control. The 5th group received sorbiline containing 550mg tricholine citrate and 7.15 gm sorbitol per 100 ml liquid (Franco-Indian) which was used as the standard drug at a dose of 1ml/kg/day for 3 weeks and the 6th group served as control and received normal saline 2ml/kg/day for three weeks. At the end of the second week carbon tetrachloride was intraperitoneally administered to all the rats except the 4th group (normal control), at a dose of 0.7ml/kg body weight, thrice a week [16]. After 48 hours of the last injection blood were collected and the animals were sacrificed by cervical dislocation.

Estimation of the enzymes SGPT and SGOT in serum
Photometric determination of SGPT.
After collecting blood from the rats of each group, serum was separated after centrifuging and immedietly taken for photometric determination of SGPT and SGOT by an Autoanalyzer of Merck (microlab 300 1X) with the help of the kit for the according to the instruction supplied with them as follows:

Principle:

$$\text{2-oxoglutarate + L-alanine} \xleftrightarrow{\text{SGPT}} \text{glutamate + pyruvate}$$

$$\text{pyruvate + NADH + H+} \xleftrightarrow{\text{LDH}} \text{Lactate + NAD+}$$

The rate of NADH consumption is measured photometrically which is directly proportional to the SGPT activity in the sample.

The reaction solution contained the following according to the supplied leaflet:

Tris buffer, pH 7.5 --------- 100 mmol/l.

L-alanine	---------	500 mmol/l.
2-oxoglutarate	---------	15 mmol/l.
NADH	---------	0.18 mmol/l.
LDH		>1.2 kU/l.

Procedure : 50 µl of serum sample was mixed wth 500 µl of reaction solution and after 1 min the absorption was measured in the following condition of the autoanalyser:

Reagent temperature : 37 °C
Wave Length : 340 nm
Light path : 1 cm
Measuring temp. : 37 °C

Calculation:
Enzyme activity [U/lt] = Abs./ minute. F [value of "F" at 340 nm is 1746]

Photometric determination of SGOT
Sample collection was same as that for SGPT.

Principle

$$\text{2-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{SGOT}} \text{glutamate} + \text{oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H+} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD+}$$

The rate of NADH consumption is measured photometrically and is directly proportional to the SGOT activity in the serum sample.
Reaction solution contained the following as per the supplied leaflet:

Tris buffer, pH 7.8	:	80mmol/l.	
L-aspartate	:	240 mmol/l.	
2-oxoglutarate	:	12 mmol/l.	
NADH	:	0.18 mmol/l.	
LDH	≥ 600 U/l.	and	MDH ≥ 420 U/l.

The procedure is same as that for SGPT

Calculation:
Enzyme activity [U/lt] = Abs./ minute. F [value of "F" at 340 nm is 1746]

Effect of TRE on normal peritoneal cells
Three groups of normal mice (n=6) were chosen for this study. The first group was administered 10 mg/kg i.p. of TRE once and the second group received such treatment for two consecutive days. The untreated third group was used as control. Peritoneal exudate cells were counted 24 hrs after treatment for each of the treated groups and compared with that of the untreated group.

Effect on functional activities of macrophages
Macrophage culture: The complete medium for macrophage culture was RPMI 1640 supplemented with FBS (heat inactivated at 56 C for 30 min)10%, 25 mM HEPES buffer, 4mM L-glutamine, streptomycin 100 microgram /ml and penicillin 100U/ml. Macrophages

were harvested from the peritoneal cavity of Balb-C mice which had been stimulated 3 days earlier by intraperitoneal injection of thioglycollate broth 4% (weight/volume).On the day of the experiment, each animal was given 20 ml of serum free RPMI medium by i.p. injection.After gentle abdominal massage,peritoneal fluid
was collected, pooled and centrifuged aseptically.The pellet was resuspended in complete RPMI medium. Cells were counted in a haemocytometer. Monolayers on glass coverslips (22 mm2) were prepared from 0.2 ml samples (1x 106 cells/ml) in 35-mm sterile petridishes [17]. They were incubated in 5% CO2 and 95% air at 37 C for 2h to allow adhesion. Non-adherent cells were removed by washing with supplemented RPMI1640 without FCS. Two ml of fresh complete culture medium was added to each petridish, and in one petridish macrophages were incubated with 10
µg/ml TRE and in another (control) macrophages with the medium only for 24hrs at 37ºC in the incubator [18,19]. After that the macrophages with extended pseudopods were counted from both the control and treated .10 ul of cell suspension was taken on slide in spreaded condition and was fixed in methanol for 5 min and then stained with giemsa (giemsa: water=1:4 v/v) for 5 min and then washed in water.Photographs were taken at 45X magnification in the Olympus Phase-contrast Compound Microscope.

Nitrous Oxide Assay on TRE activated Macrophages

Since it was found that isolated peritoneal macrophages were extending pseudopods after being treated with TRE, it was necessary to investigate the functional activity of the macrophages and so it was investigated as to whether nitrous oxide synthesis was being increased in macrophages in presence of TRE. 105/ml macrophagic cells isolated as mentioned above] in RPMI 1640 media with 10% FCS were taken in 35 mm2 petridishes and were treated with of 5, 10 & 15 µg/ml TRE for 24 hrs and then NO assay was done using Griess reagent and the optical density (O.D.) was measured at 560nm.

Statistics

Data are presented as arithmetic mean ± S.E.M. of at least 6 experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test or by Student's paired‘t’ test. "p" value of less than 0.05 was considered as statistically significant.

3. Results and Analysis

Acute toxicity studies

The LD_{50} of TRE was found to be 100 mg/kg i.p.

Anti-inflammatory studies Carrageenan-induced oedema

TRE inhibited carrageenan-induced paw oedema by 41% in volume and 50% in weight which was slightly more than that produced by acetyl salicylic acid and highly significant as compared to control (**Table 1**).

Table 1. Effect of TRE on carrageenan-induced paw oedema in rat

Drug	Dose	Difference in paw volume in ml (mean ± S.E.M.)	Difference in paw weight in gm(mean ± S.E.M.)
Saline control	0.1ml/100g(i.p.)	4.6 ± 0.41	0.46 ±0.017
Acetyl salicylic acid	100mg/kg(i.p.)	2.8 ± 0.42*	0.36 ± 0.010*
TRE	10mg/kg(i.p.)	2.7 ± 0.20*	0.23 ±0.007*

Data were analysed by ANOVA and Dunnett's test.

*Denotes significant inhibition as compared to saline control ($p<0.05$)

Freund's adjuvant induced polyarthritis

Prophylactic treatment with TRE (10 mg/kg i.p.) inhibited development of arthritis by 49% as detected by reduction of paw volume (**Table 2**) and increased mobility of joints. TRE also inhibited secondary lesions at this stage. TRE also showed 11% more inhibitory effect as compared to acetyl salicylic acid. The secondary lesions like tail and ear buds as well as joint movements were also inhibited after 21 days consecutive treatment with TRE as compared to control.

Table 2.Effect of TRE on Freunds Adjuvant induced polyarthritis

Drug	Dose	Difference in the paw volume between day-0 and day-21 in ml (mean ± S.E.M.)
Saline control	0.1ml/100g(i.p.)	1.58 ± 0.27
Acetyl salicylic acid	100mg/kg (i.p.)	0.90 ± 0.37*
TRE	10mg/kg (i.p.)	0.80 ± 0.12*

Data were analysed by ANOVA and Dunnett's test.

*Denotes significant inhibition as compared to control ($p<0.05$).

Effect on isolated rat diaphragm

TRE at all concentrations (5, 10 and 15 mg/ml in the 10 ml organ bath) produced an insignificant blokade of indirect twitch responses of rat diaphragm preparation .When less than 5mg/ml was used, TRE failed to alter the indirect twitch response and at higher concentrations also it did not cause any more inhibition or facilitation.TRE at a dose of 5mg/ml produced 18.15% inhibition ,10 mg/ml caused 26.76% inhibition and 15 mg/ml caused 24.46% inhibition as compared to general twitch height of preparations in Kreb's solution.The statistical analysis of the effect is presented in **table-3** and % inhibition is given graphically in **figure-1** and the recording in **figure-2.**

Table-3. Effect of TRE on indirect twitch responses of rat diaphragm preparation.

Condition of the bath	Twitch height in inches(mean ± S.E.M.) [n=6]
Kreb's solution	1.68 ± 0.24 (inhibition)
TRE (5 mg/ml)	1.4 ± 0.22 (inhibition)
TRE (10 mg/ml)	1.3 ± 0.23 (inhibition)
TRE (15 mg/ml)	1.3 ± 0.21 (inhibition)

FIG-1 :-Percentage Inhibition of Indirect Twitch Response of Rat Diaphragm Preparation by TRE at Different Doses

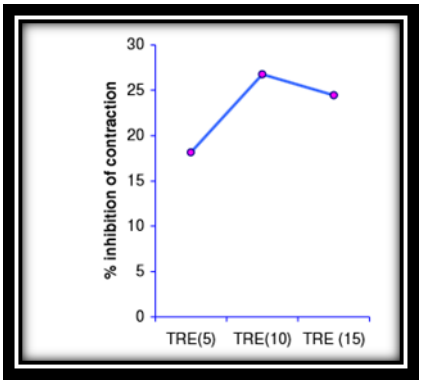
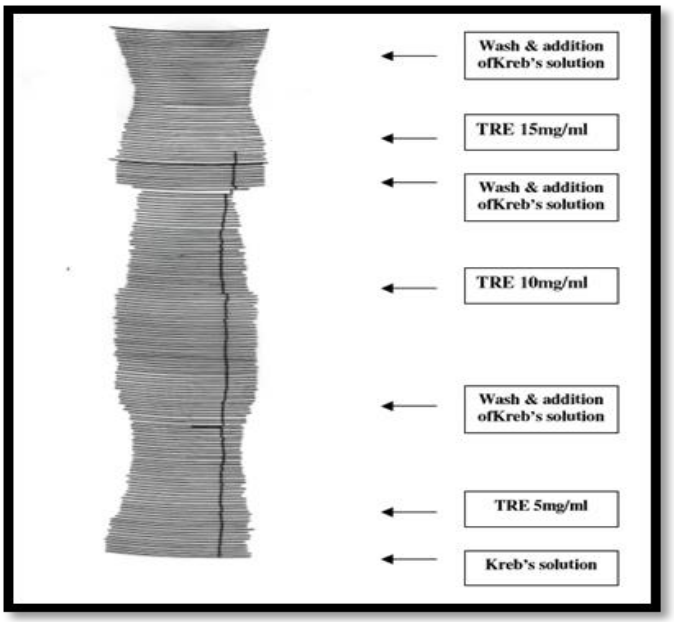


Fig-2-Diagram of the recording of twitch response of rat diaphragm in normal bath and in presence of TRE



Effect on CCl4 induced hepatotoxicy

General histology

Histopathological profile of liver of carbon tetrachloride treated rats showed vacuolisation and centrilobular necrosis. Liver of the rats treated with sorbiline showed significant signs of amelioration of carbon tetrachloride induced liver injury as evident from the presence of normal hepatic cord, absence of necrosis and vacuoles.TRE in all the three doses showed very less effect and the recovery of the tissue necrosis was also negligible but the continuous treatment did not cause any increase in the tissue necrosis which reveals the fact that TRE may not be hepatoprotective but it was not hepatotoxic in that way.

Level of SGPT: There was significant increase in the serum GPT level in the carbon tetrachloride induced hepatotoxic group as compared to normal rats. In the TRE treated group all the three doses mentioned above reduced the serum GPT level insignificantly.The sorbiline treatment only caused significant reduction in SGPT level as compared to carbon tetrachloride control (**Table-4 and Figure-3).**

Level of SGOT: The serum GOT level was almost doubled in CCl4 treated copntrol group as compared to that of the normal rats and treatment with the standard drug sorbiline reduced the level significantly toward normal value. TRE treatment caused insignificant reduction in the enzyme level as compared to control (**Table-5 and Figure-3**).

Table-4

SGPT level (expressed as Unit/Lt) of the rats of different groups

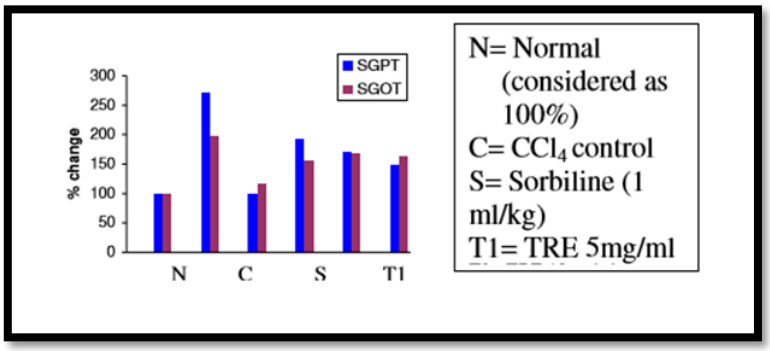
Groups(n=5)	Mean±S.E.M.(unit/l.)
Normal(without CCl_4)	50 ± 1.73
Control(with CCl_4)	135.52 ± 14.33
TRE 5mg/ml (with CCl_4)	96.9 ± 4.68
TRE 10 mg/ml (with CCl_4)	84.94 ± 7.37
TRE 15 mg/ml (with CCl_4)	73.8 ± 2.73
Sorbiline(with CCl_4)	50.12 ± 4.42*

Table-5

SGOT level (expressed as Unit/Lt) of the rats of different groups

Groups(n=5)	Mean±S.E.M.(unit/l.)
Normal(without CCl_4)	101.28 ± 2.95
Control(with CCl_4)	200.42 ± 17.27
TRE 5mg/ml (with CCl_4)	158.06 ± 10.20
TRE 10 mg/ml (with CCl_4)	170.98 ± 6.49
TRE 15 mg/ml (with CCl_4)	164.9± 9.26
Sorbiline(with CCl_4)	118.82 ± 10.5*

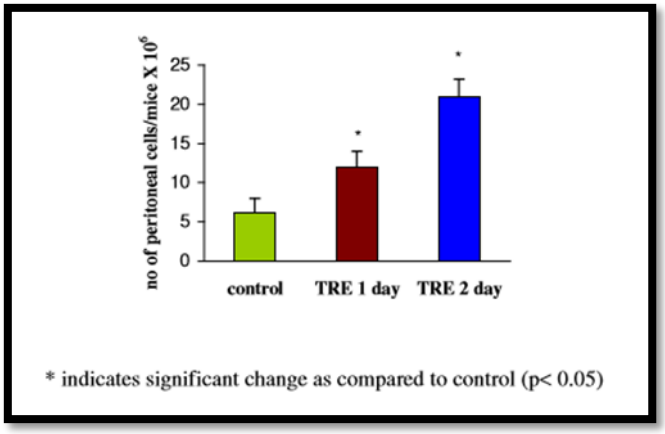
Figure-3: Histogram showing the % change in the Serum GPT and GOT level of CCl4 treated rat with TRE Treatmentconsidering the level of the enzyme in normal as 100%.



Effect on normal peritoneal cell

The average number of peritoneal exudate cells in control rats was found to be 6.2 ± 1.8×10^6. TRE (10 mg/kg i.p.) treatment increased the no. of peritoneal cells to $12 \pm 2 \times 10^6$ in single treatment and to 21 ± 2.2 × 106 in two when treatment was done for two days (Figure-4).

Figure-4: Increase in peritoneal exudate cells of mice after TRE treatment



Effect on the Macrophages Count of Macrophages with extended pseudopods TRE did not increase the number of macrophages in the in vitro condition after 24 hrs but it significantly increased the the number of active macrophages with extended pseudopods.TRE at a dose of 15 µg/ml increased the number of active macrophages to 52.5 % as compared to control which reflects the immunomodulatory activity of TRE (Figure 5 and 6).

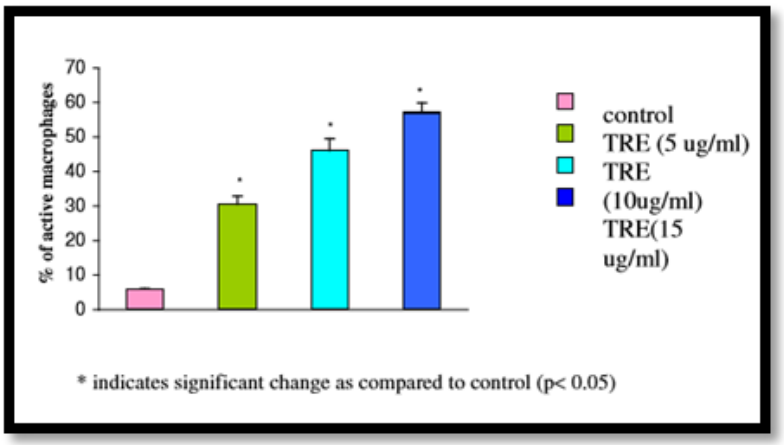
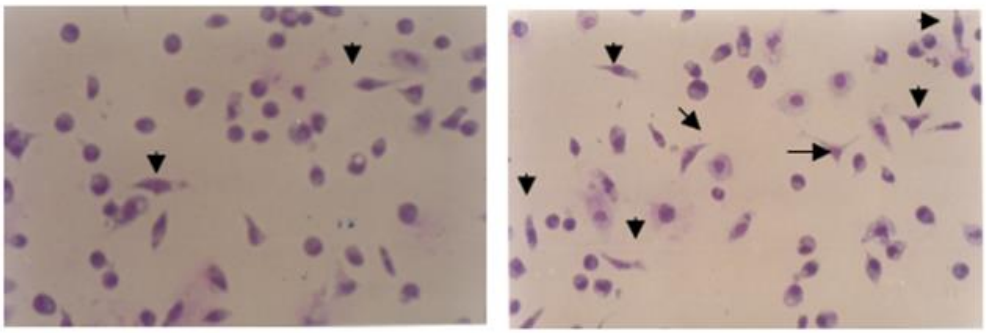


Figure-5: Percentage of Active Macrophages After TRE Treatment in *in vitro* condition



Control Treated

Fig:6 The macrophages with arrowheads indicate extended pseudopodes

Effect on NO Synthesis: It was found from the experiment that TRE at a dose of 15µg/ml caused an increase in the production of nitrous oxide by the macrophages upto 32% (**Figure7)**.

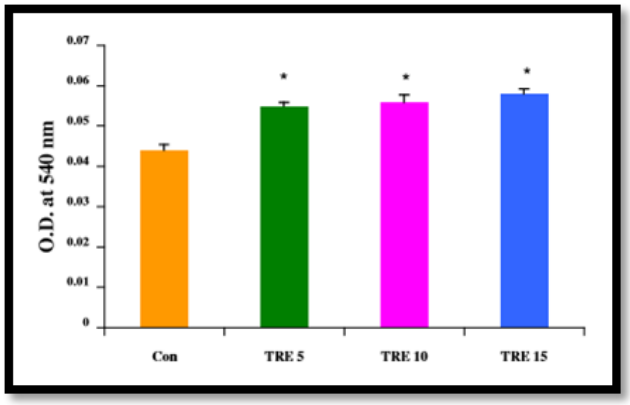


Figure-7:Nitrous Oxide Synthesis by Activated macrophages After Treatment with TRE

Conclusion

The present study revealed that TRE possesses significant anti-inflammatory activities in experimental animals at a dose of 10 mg/kg body weight (i.p.) which is one-tenth of its LD_{50} dose of 100mg/kg i.p. The anti-inflammatory effect of TRE was observed in acute and chronic models of inflammation. The observation that TRE significantly reduced inflammation in the Freund's adjuvant induced polyarthritis in rat reveals that TRE possesses antiarthritic activity as well.

It is well known that there is a close relationship between inflammation and cancer [20,21]. It has been reported that tumour promoters recruit inflammatory cells to the application site and cancer development may also act by aggravating inflammation in the tissue and vice versa and that inflammatory cells are capable of inducing genotoxic effects [22]. So it is likely that anti-inflammatory agents may possess antitumour activity and vice versa. Since it has earlier been reported that TRE possesses anti-tumour effect in ascites [23] and solid tumour [24], the present observation of TRE possessing significant anti-inflammatory activity provides further support for such a contention

The study on the effect of TRE on skeletal muscle and its neurotransmission revealed that though TRE caused mild inhibition in the indirect twitch response the percentage inhibition was insignificant which means that TRE does not cause any paralytic effect on the muscle contraction in general i.e. TRE is not neurotoxic in that way. In many Cases as it is found that many life-saving drugs cause contractile dysfunction of many muscles. It was found that Zidovudine (AZT), a primary drug, used to treat HIVinfected individuals, induced a drug specific myopathy resulting in altered muscle function and increased oxidative stress in the muscle which could be considered as a harmful side effect [25]. It is also reported that Senna occidentalis, used for many medicinal purposes possesses highly toxic effects on the central nervous system and can cause degeneration and spongiogenesis for which despite of its several beneficial activities it is risky to use it for general medicinal purpose [26]. Researches made on antioxidants have shown that antioxidants, which are specially superoxide scavengers, protect contractile function in severe hypoxia [27].

It is well established that the hepatotoxicity by CCl_4 is due to its enzymatic activation to CCl_3, which in turn disrupts the structure and function of the lipid and protein macromolecules in the membranes of the cell organelles [28,29] and induces microsomal lipid peroxidation leading to fatty liver . Our present studies have shown that TRE was unable to reverse the toxicity and damages caused by CCl_4 significantly as revealed by the SGPT and SGOT levels in rats. However in TRE treated rats the enzymatic level was much lower than that of the control or untreated CCl_4 induced hepatotoxic rats which indicated that though TRE does not possess hepatoprotective, by itself it does not possess any hepatotoxic effect as well. Many herbal therapeutic agents like Pyrrolizidine alkaloids cause obstruction of the hepatic.

To evaluate whether TRE treatment has any effect on the cell growth, the effect of TRE was evaluated on the peritoneal exudate cells of normal mice. Normally each mouse contains about 5×10^6 intraperitoneal cells (similar to the observation in the present study), 50% of which are macrophages. TRE treatment enhanced the peritoneal cell count and also the number of macrophages. Non-specific accumulation of macrophages occurs in the peritoneal cavity after injection of certain materials such as casein, Freund's complete adjuvant, thioglycolate, etc. Mature macrophages in the untreated peritoneal cavity are mostly residential. Intraperitoneal injection of different agents leads to exudation and intraperitoneal accumulation of new macrophages, which differ from mature macrophages. It has been reported that generally the exudate macrophages are more active than the residential mature ones in their ability to spread on the surface to which the cells are attached, receptor size of cell coat, response to chemotactic stimuli, composition of cell wall, etc. . Though the actual role of TRE in the enhancement of peritoneal cell count and macrophage count cannot be explained at the present juncture, it is possible that TRE may also alter the immune response along with its anti-inflammatory effect.Overall the present investigation establish the promising therapeutic potential of the tea root extract in future as a herbal drug in treating inflammation and cancer with comparatively negligible side effects.

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