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Original Article

# Assessment of Anticarcinogenic Potential of *Vitex trifolia* and *Triticum aestivum* Linn by *In Vitro* Rat Liver Microsomal Degranulation

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## ABSTRACT

**Objective**: The main objective of this preliminary study is to confirm the synergistic anticarcinogenic potential of *Vitex trifolia* and *Triticum aestivum* ethanolic extracts. **Materials and Methods:** Rat hepatic microsomal degranulation is a short - term technique that has been used for the detection of potential chemical carcinogens, *in vitro.* The present study has been carried out to study the inhibition of ribosome- membrane disruption against 3, 8-Diamino-5-ethyl-6-pheylphenanthridinium bromide (EB), as the degranulating agent, by measuring the RNA/protein ratios of microsomal membranes in the presence or absence of *V.trifolia* and *T. aestivum* extracts. These two extracts were further evaluated for cytotoxic effect in HCT 116 and A549 cell lines. **Results:** *V. trifolia* and *T. aestivum* protects hepatic microsomes against the degranulatory attack by the carcinogen EB showed a significant reduction in the proliferation of the HCT 116 and A549 cancer cell lines. **Conclusion:** The ethanolic extracts of the plants, *V. trifolia* and *T. aestivum* individually possessed anti-degranulatory potential. Importantly they act synergistically, possess appreciable anticarcinogenic properties, based on their ability to inhibit EB induced liver microsomal degranulation.Further these extracts inhibit cell proliferation of cancer cell lines.

**Key words:** 3,8-diamino-5-ethyl-6-phenylphen-anthridinium bromide, degranulation, *Vitex trifolia*, *Triticum aestivum*

## INTRODUCTION

Herbal and food - based medicines obtained from plant extracts have been traditionally utilized to treat a wide variety of diseases and clinical disorders and yet relatively little knowledge is available about their mode of action, specific molecules involved, and exact effective doses. For instance, plants that are rich in polyphenolic compounds, especially flavonoids, are used to benefit clinically human health by reducing the incidence of cancer.[1] The plant *Vitex trifolia* L., (*Verbenaceae*) is a flavonoid rich medicinal plant which has been traditionally used to treat rheumatic pain and inflammation.[2] Also, pharmacological properties have been linked with *Vitex* viz., antibacterial.,[3] and hepatoprotective activity.[4] Recently the flavonoids have been shown as novel cell cycle inhibitors.[5] The shoot of *Triticum aestivum* L., (Wheat grass) contains flavonoids which possess anti-cancer activity, ulcer-alleviating

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**Access this article online**

Quick Response Code:

Website: www.toxicologyinternational.com

DOI: 10.4103/0971-6580.172269

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**How to cite this article:** Mathankumar M, Tamizhselvi R, Manickam V, Purohit G. Assessment of anticarcinogenic potential of *Vitex trifolia* and *Triticum aestivum* Linn by in vitro rat liver microsomal degranulation. Toxicol Int 2015;22:114-8.

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ability, antioxidant activity, anti-arthritic activity, and blood refreshing activity in Thalassemia Major. The key clinical utilization of wheat grass might be due to the presence of high content of bioflavonoids such as apigenin, quercitin, luteoline. Furthermore, indole compounds namely choline and laetrile present in it might also be responsible for its therapeutic potential.[6] Since very few studies have been made on these very promising herbal drugs, it was thought worthwhile to study the anticarcinogenic effect of *V.trifolia* and *T. aestivum* against a provenchemical carcinogen by a short - term technique, under *in vitro* conditions, using rat liver microsomes. Also, synergistic actions of these extracts were investigated in this study.

## MATERIALS AND METHODS

### Preparation of the extract

Leaves of V. *trifolia* L., (*Verbenaceae)* and Shoot of *T. aestivum* L were collected from VIT university plant nursery, Vellore, Tamil Nadu. India during the month of September and were identified by the botanist, Professor Dr. Ayyanar, Loyola college, Chennai, India. The *V. trifolia* leaves and *T.aestivum* shoots were shade-dried for 1 week and ground into fine powder. The ground leaf powder (25g) was extracted with 250 ml of 90% ethanol in soxhlet apparatus. The ethanolic mixture was transferred into rotary evaporator for removal of solvent from sample. The extract (around 5 g) was stored at −20°C before assay.

### Chemicals

The chemicals used in the present study were purchased from Sigma-Aldrich (India) and S. D. Fine Chem Ltd and were of analytical grade.

### Animals

Male Wistar rats, weighing 120-150g were used. The animals were cared in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision on Experiments on Animals and the Institutional Animal Ethics Committee approved the entire study (Approval no.VIT/IAEC/VII$^{th}$/34/2013).

### Estimation of total phenolic content

Total phenolic content was determined by reaction with Folin-Ciocalteu reagent, as described by Singleton and Rossi[7] and Kahkonen *et al.*[8] Leaf extract (1mg/ml) was mixed with 2 ml of Folin-Ciocalteu reagent (which had previously been diluted 10-fold with distilled water) and allowed to stand at room temperature for 8 min; 1.6 ml of 7.5% $Na_2CO_3$ solution was added to the mixture. Contents of the tubes were mixed and allowed to stand in the dark for 30 min, after which the absorbance was measured at 765 nm (Hitachi U-2001). The results were expressed as gallic acid equivalents per 100 mg of crude extract (mg gallic acid equivalents/100 mg extract).

### Estimation of flavonoid content

Flavonoid content was determined by the procedure described by Zhishen *et al*.[9]

Leaf extract (1mg/ml) was mixed with 0.3 ml of 5% aqueous $NaNO_2$ (w/v) and allowed to stand at room temperature for 5 min; 0.6 ml of 10% AlCl3 solution (w/v) was added to the mixture. 2 ml of 1M NaOH and 2.1 ml of water were added to the mixture after 6 min. The absorbance was measured at 510 nm (Hitachi U-2001). The results were expressed as quercetin (Q) equivalents per 100 mg of crude extract (mg Q/100 mg extract).

### Microsomal degranulation assay

Five rats were sacrificed for each experiment and their livers were chopped. To 0.5gm of liver 1.25 volume of 0.225 M sucrose tris (ST) buffer (pH 7.4) were added and the mixture was homogenized in chilled condition and processed for microsomal degranulation.[10,11] Tissue homogenates were centrifuged for 20 min at 9000 rpm at 4°C, the post mitochondrial supernatant collected and mixed with 0.5 g calcium chloride. After that the tubes were kept in ice for 20 min, centrifuged at 4°C, 10,000 rpm for 20 min. The pelleted microsomes were resuspended in 0.225 M ST buffer (pH 7.4) and protein[12] and RNA[13] were estimated as per the standard methods.

### 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Cell viability was determined by using [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] MTT assay according to a previously described protocol.[14] HCT116 and A549 cells, both cancer cells originated respectively from lung and colon cancer, were harvested by trypsinization and resuspended at a final concentration of $2 \times 10^4$ cells/ml in fresh DMEM with 2% Fetel bovine serum. Aliquots of 100 µl cell suspension were plated in 96-well tissue culture plates. In order to detect the cytotoxicity of the cells, cells were treated with *V.trifolia* at a concentration range 20, 40, 60 and 80µg/ml together with 100, 200, 400, 500µg/ml of *T. aestivum* for 24 hours. After 24h, 20 µl of a 5 mg/ml MTT solution was added to each well, and the plate was incubated for 4h, allowing viable cells to reduce the yellow MTT to dark-blue formazan crystals, which were dissolved in 100 µl of DMSO. The absorbance in the individual well was determined at 570 nm using microplate reader [ELx-800 biotek absorbance reader]. The cell

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viability was calculated as percentage of viable cells and then plotted on a graph.

Growth inhibition (%) = (A570 nm of treated cells/A570 nm of control cells) ×100

### Statistical analysis

Data are expressed as the mean ± standard deviation of the mean (SD).

## RESULTS

### Phenolic and flavonoid content

We initially estimated the phenolic and flavonoid content of *V. trifolia* and *T. aestivum* as these compounds may contribute directly to the net anticarcinogenic potential of leaf extract. Accordingly, the total phenolic content of *V. trifolia* was 0.805 ± 0.04 mg gallic acid per 100 mg extract and *T.aestivum* was 0.705 ± 0.022 mg gallic acid per 100 mg extract [Table 1]. Similarly the flavonoid content of the extracts were investigated to explore the quenching ability of *V. trifolia* and *T. aestivum* in reducing the oxidative damage to cells as demonstrated before.[9] The total flavonoid content of *V. trifolia* was 0.487 ± 0.035 mg quercetin equivalent per 100mg extract and *T. aestivum* was 0.305 ± 0.022 mg quercetin equivalent per 100mg extract [Table 1].

### Microsomal degranulation

The observations recorded indicate that 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (EB) is capable of inducing microsomal degranulation extensively ($P < 0.001$) (62% on the basis of RNA/protein ratio) [Table 1]. Application of whole extract from *V. trifolia* shows 85% protection against EB mediated degranulation whereas *T. aestivum* shows 75% protection ($P < 0.001$). Cumulatively both *V. trifolia* and *T. aestivum* shows 91% protection ($P < 0.001$) [Table 2].

### Antiproliferative activity

Basically, MTT proliferation assay is based on the ability of viable cells with active mitochondrial dehydrogenase enzyme which cleave the tetrazolium rings of MTT where the optical density obtained was proportional to the number of healthy viable cells. In this study, HCT116 and A549 cancer cells were treated with *V. trifolia* at a concentration range 20, 40, 60 and 80µg/ml together with100, 200, 400, 500µg/ml of *T. aestivum* for 24 hours. Based on the graph plotted, IC50 value was determined.Figure 1 shows, the IC50values after 24 hour treatment with a combination of40µg/ml *V.trifolia* and 200 µg/ml *T.aestivum*. The synergic combination of these two extracts showed a significant reduction in the proliferation of A549 and HCT116 cell lines.

**Table 1: Concentration of total phenolic compounds in gallic acid equivalents and flavonoids in quercetin equivalents in GLE**

| Plant extract | Total phenolic content (mg gallic acid/100 mg) | Total flavonoid content (mg quercetin equivalent/100 mg) |
|---|---|---|
| *Vitex trifolia* | 0.805±0.04 | 0.487±0.035 |
| *Triticum aestivum* | 0.705±0.02 | 0.305±0.022 |

**Table 2: Hepatic microsomal degranulation by EB and its inhibition by *Vitex trifolia* and *Triticum aestivum***

| Composition of incubation mixture | RNA/protein±SD *n*=5 | Percentage of degranulation (D) and protection (P) |
|---|---|---|
| Microsomes | 0.410714±0.108972 | 0 (D) |
| Microsomes + EB + Pmics | 0.234524±0.088736* | 42.89855 (D) |
| Microsomes + *Vitex trifolia* + Pmics | 0.408333±0.114972* | 99.42029 (P) |
| Microsomes + EB + *Vitex trifolia* + Pmics | 0.352381±0.090445# | 85.7971 (P) |
| Microsomes + *Triticum aestivum* + Pmics | 0.352381±0.243076* | 85.7971 (P) |
| Microsomes + EB + *Triticum aestivum* + Pmics | 0.25119±0.06148# | 75.81159 (P) |
| Microsomes + *Vitex trifolia* + *Triticum aestivum* + Pmics | 0.397857±0.225623* | 98.04348 (P) |
| Microsomes + EB + *Vitex trifolia* + *Triticum aestivum* + Pmics | 0.37619±0.005455# | 91.5942 (P) |

Values are mean±SD, *n*=5. *$P$<0.001 when compared with microsomes, #$P$<0.001 when compared with microsomes + EB + Pmics. Pmics = Postmicrosomal supernatant, SD = Standard deviation, EB = 3, 8-diamino-5-ethyl-6-pheylphenanthridinium bromide

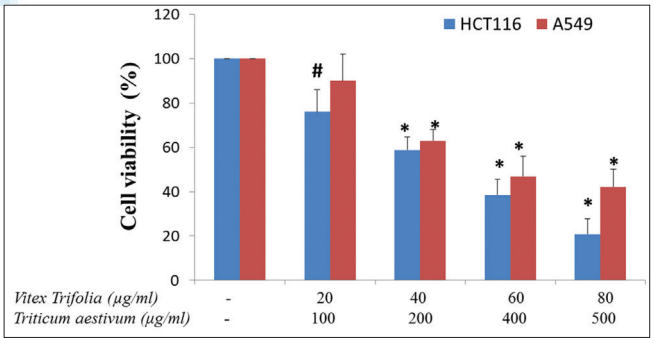



**Figure 1:** Inhibition of A549 and HCT-116 cell growth by *V.trifolia* and *T. aestivum* as determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Cells were exposed for 24 h to the indicated concentrations of *V. trifolia* and *T. aestivum*. Values are the mean from three experiments; bars, standard deviation *$P$ < 0.001 when compared with control; #$P$ < 0.01 when compared with control

### Statistical analysis

Data are expressed as the mean ± (SD) of the mean. Vertical error bars denote the SD. The significance of differences between groups was evaluated by analysis of variance with *post hoc* Tukey's test when comparing three or more groups. A $P < 0.05$ was considered statistically significant.

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## DISCUSSION

In the present study the model utilizing the carcinogenic potential of (EB) was assessed by measuring the detachment of ribosomes from rough endoplasmic reticulum (RER). Earlier studies have reported that carcinogens degranulated RER under *in vivo* and *in vitro* conditions [15,16] resulting in a decreased RNA/Protein ratio and provides the basis of ascreening test for environmental or chemical carcinogens.[17] Liver provides a good model for the study of carcinogen-induced degranulation, as it is a rich source of RER and it has the metabolic transformation capacity required to generate active forms of electrophilic carcinogens from precursors. In the present study *V. trifolia* and *T. aestivum* exposure, both individually and when given together, resulted in an increased RNA/Protein ratio which has been taken as an index of protection of microsomal degranulation. Our results are in accordance to the earlier findings[18] an increase in RNA/Protein ratio of treated rats due to direct protection of membrane degranulation has been reported. Researchers have demonstrated that electrophiles of a carcinogen cause degranulation (removal of ribosomes) from RER, involved in protein synthesis for export from cytosol. Ribosome loss is monitored by determining RNA/protein ratio for membranes due to loss of RNA with the ribosomes.[19] Detachment of ribosomes from the extravesicular surfaces of RER may lead to alterations in the pattern of cellular protein synthesis and alteration in gene expression leading to carcinogenesis. In this study we found that *V.trifolia* and *T. aestivum* significantly inhibited carcinogen induced microsomal degranulation which points to the negative regulatory role of these extracts during the carcinogenesis process. It has been reported that the plant flavonoids showed marked reduction in the degree of ribosomal detachment from the microsomes.[20]

Moreover, treatment with combination of *Triticum* and *Vitex* extract was found to inhibit cell proliferation in HCT116 and A549 cancer cells. From our findings, it is clear that the flavonoids present in the *Triticum and Vitex spp* possess considerable anticarcinogenic role. *V. trifolia* (80mg/ml)[21] and *T. aestivum* (600mg/ml)[22] has been reported for its anticarcinogenic properties. Synergistic effect showed that the addition of *T.aestivum* extract appreciably enhanced the anticarcinogenic potential of *V. trifolia*. This is the first report to show the synergistic effect of these herbs and the finding could have very important applications in the design of functional foods and in the exploration of novel lead moieties against cancer and other disorders. Thus, in phytotherapy, there are potentially significant advantages associated with the synergistic interactions of different plant extracts such as increased efficiency, reduction of undesirable effect, increase in stability and obtaining an adequate therapeutic effect with relatively small doses, when compared with a synthetic medication.[23]

## CONCLUSION

Also to the encouraging anti-carcinogenic activity of individual extracts using microsomal degranulation studies, when combined at the whole extract level, there exists enhanced synergy between *V.trifolia* and *T. aestivum*. Our study highlights the mechanistic role viz., hepatoprotective, anti-carcinogenic prospective of these plants extracts and their application in traditional medicines. Interestingly the enhanced level of anti-carcinogenic activity confirms the unexplored anti-cancerous potential behind these customary formulations. Also there is an urgent need for more studies concerning the molecular basis of synergistic interactions, and to understand the individual component in the development of pharmacological agents to treat cancer and other chronic inflammatory disorders. Fulfilling these goals could bring more druggable lead molecules from the traditional medicinal formulations followed with ancient Chinese and Indian treatment modalities. Incidentally these treatment measures are effective only when given as the combinatorial formulation, not as the individual entity.

### Financial support and sponsorship

The authors are grateful to VIT University, Vellore, Tamilnadu, India for providing financial support for this investigation.

### Conflicts of interest

The authors have declared that there is no conflict of interest.

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