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# Research Article
# Supplementation of 500 mg kg$^{-1}$ Tocotrienol-Rich Fraction (TRF) in Mice Induced Alteration in Liver Protein Expression

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## Abstract

**Background and Objective:** The most popular tocotrienol preparation, tocotrienol-rich fraction (TRF), offers the best biological and antioxidant properties. This investigation aims to ascertain the impact of TRF on the overall expression of proteins in the liver of mice. **Materials and Methods:** Two groups of mice were created: A control group (n = 6) and a group treated with 500 mg kg$^{-1}$ (n = 6). Following a 14-day course of therapy, the mice were killed and their livers were separated. After the livers were homogenised, Two-Dimensional Gel Electrophoresis (2-DE) was performed on the soluble cytosolic fractions. The 2-DE gels stained with Coomassie Blue were scanned and 2-DE gel spot analysis software was used to examine the resulting images. Twelve gels in total were examined (six for every group). **Results:** For every gel, about three hundred spots were detected. Differential expression of twelve protein spots was observed in the livers of mice treated with a tocotrienol-rich fraction. Five of the proteins are acidic proteins, while seven of them are basic proteins. The TRF downregulated three protein spots and upregulated nine protein spots. These findings suggest that TRF profoundly influences several proteins and simultaneously regulates a multitude of biochemical pathways. **Conclusion:** It is hypothesized that some of the proteins that are differentially expressed might be the liver antioxidant proteins and cytoprotective enzymes. Further analysis of the gel spots by mass spectrometry is needed to identify the proteins involved.

Key words: Proteomics, Two-Dimensional Gel Electrophoresis (2-DE), vitamin E, tocotrienol-rich fraction (TRF), mice, liver

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

A significant class of chemicals having a variety of biological effects is vitamin E. By scavenging free radicals in the early phases of free radical attack, vitamin E is widely acknowledged as the first line of antioxidant defence against lipid peroxidation, safeguarding polyunsaturated fats in cellular membranes[1]. A total of eight distinct isoforms of vitamin E that belong to two families are known to occur in nature; α, β, γ, δ tocopherols and α, β, γ, δ tocotrienols. These substances have an isoprenoid side chain and a 6-chromanol ring structure[2]. Tocotrienol rich fraction (TRF) is the term used to describe an isolated fraction of palm oil that mostly consists of α, γ, δ-tocotrienols and some α-tocopherols[3]. The majority of tocopherols have saturated side chains, whereas the side chains of tocotrienols have double bonds at the 3', 7' and 11' locations. A significant portion of the overall vitamin E content found in annatto, rice bran and palm oil are tocotrienols[4]. The antioxidant activity of tocotrienols has encouraged many researchers to study its ability to cure chronic diseases, especially those in which oxidative stress is implicated[5]. Tocotrienols have been reported to have anticancer properties through both animal and cellular studies[6]. For many years, α-tocopherol was widely considered to be the most potent antioxidant against lipid peroxidation in the vitamin E group. However, tocotrienols have been reported to possess better antioxidant properties than α-tocopherol. Palm oil, a major product of Malaysia, is a very rich source of tocotrienols[7].

Experimental studies both *in vitro* and *in vivo* have indicated that tocotrienols prevent the growth and proliferation of many cancerous cells. Previous animal studies had indicated that tocotrienols might have chemopreventive properties[8,9]. Tocotrienols were chemopreventive in the rat liver tumour model[10,11]. Tocotrienols have also recently been observed to show chemopreventive effects against HepG2 liver cancer cell line[12].

The mechanisms responsible for the chemopreventive effects of tocotrienols are not fully understood. A few possible mechanisms that have been hypothesized include induction of antioxidant activity, suppression of HMG-CoA reductase activity, pro-apoptotic effect as well as potential anti-angiogenic activity. It has also been suggested that tocotrienols prevent the Akt and nuclear factor kappa B activity, leading to down-regulation of various gene products and potentiation of apoptosis[5,6]. However, these postulated mechanisms were elucidated from cells other than the liver (in particular breast cancer cell lines) and the exact pathways involved have not been fully characterized. To further explain the mechanisms involved in the antioxidative and chemopreventive effects of tocotrienols (especially in the liver) it would be of interest to identify liver proteins whose expressions are affected by exposure to tocotrienols, using animal models.

Tocotrienols specifically δ- and γ-tocotrienol (GTT) modify cellular redox status by influencing the expression levels of antioxidant enzymes. The GTT inhibits the proliferation of MCF-7 cells while upregulating the expression of several antioxidant enzymes, including thioredoxin and quinone reductase 2 (NQO2)[13]. Tocotrienol administration resulted in several-fold upregulation of NRF2 expression in MDA-MB-231 cells, accompanied by a comparable downregulation of Kelch-like ECH-Associated Protein 1 (KEAP1) levels[13]. Treatment of HepG2 cells with GTT was also observed to elevate the expression of Peroxiredoxin-4 (Prx4), an antioxidant protein[12]. Major intracellular antioxidants involved in our body's detoxification processes include glutathione peroxidase, superoxide dismutase and catalase[14].

Proteomics is a robust method that allows researchers to profile cellular responses to various physiological and pathological conditions at the protein level. It has been utilized as a tool in cancer research, particularly in the search for new biomarkers[15]. Apart from that, proteomics has also been used in the analysis of the response of the liver towards xenobiotics[16]. Using the Two-Dimensional Gel Electrophoresis (2-DE) technique it was observed that 18 protein spots were differently expressed in HepG2 hepatoma cells treated with tocotrienols, a very small number indeed because about 1000 protein spots can be detected in a single conventional 2D gel of the liver[17]. Previous studies indicated that tocotrienols might have chemoprotective properties, therefore, it is interesting to discover the range of proteins affected by TRF supplementation especially in the liver, using the Two-Dimensional gel (2-DE) proteomics technique. In this study, the 2-DE method was used to elucidate changes in protein expression in mice liver after supplementation with 500 mg $kg^{-1}$ TRF, compared to controls. The dose of 500 mg $kg^{-1}$ TRF was chosen because, in previous studies, 500 mg $kg^{-1}$ TRF was able to cause a significant increase in the expression of individual hepatoprotective proteins such as NQO1, HO1 and GSTs[18-20]. Therefore, the purpose of this study is to identify mice liver proteins that are upregulated and downregulated after supplementation of 500 mg $kg^{-1}$ TRF to mice for 14 days using the 2-DE proteomics method, which would provide a better perspective on the effect of tocotrienols on global liver protein expression.

## MATERIALS AND METHODS

**Study area:** The current study was carried out from July to November 2020 at the Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia Cheras Campus, Kuala Lumpur, Malaysia.

Table 1: Running conditions for isoelectric focusing

| Conditions | Voltage (V) | Time (hrs ) |
|---|---|---|
| 1 | 150 | 1 |
| 2 | 300 | 1 |
| 3 | 1500 | 1 |
| 4 | 3000 | 18 |

**Animal handling and treatments:** Twelve Institute of Cancer Research (ICR) male white mice aged between 10-12 weeks (25 to 30 g) were obtained from Universiti Kebangsaan Malaysia Animal Laboratory Unit. Mice were housed in clean polypropylene cages with food and water available *ad libitum*. The cages were placed in a well-ventilated room with a 12 hrs light-dark cycle. The mice were divided into two groups i.e., the TRF-treated group and the control group (n = 6 for each group). The TRF group received TRF (500 mg/kg/day) dissolved in corn oil via oral gavage for 14 days. The control group was only given the vehicle (corn oil) via oral gavage. The TRF (Gold Tri.E 70) was obtained from Sime Darby Oils Nutrition, Malaysia.

**Ethical consideration:** The use of animals in this study has been approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with approval number: FAR/FP/2020/AZMAN/14-MAY/1105-JULY.-2020-DEC.-2021-NAR-CAT2.

**Sample preparation for 2-DE:** The mice were sacrificed on day 15. Liver tissues were excised and immediately snap-frozen in liquid nitrogen. The specimens were stored at -80°C until further analysis. About 50 mg of the liver was used for extraction. Protein extraction was carried out using commercialized protein extraction kits, the ProteoExract® Native Membrane Protein Extraction Kits (MPEK), Merck KGaA, Darmstadt, Germany. The procedure of the extraction was based on the insert kit provided by the manufacturer. Protein quantification was determined using colorimetric assay using (bicinchoninic) Novagen BCA Protein Assay Kit (71285-3) Merck KGaA, Darmstadt, Germany.

**Two-Dimensional Gel Electrophoresis (2-DE):** The first dimension was performed using immobilized pH gradient (IPG) strips with properties of 13 cm, non-linear, pH 3-10 (GE Healthcare, UK) on BioRad Protean 112 IEF Cell. The strips were first passively rehydrated overnight at room temperature with the rehydration buffer (7 M Urea, 2% CHAPS, 2% IPG buffer, 2M thiourea and 0.0025% bromophenol blue) together with 500 µg of mice liver cytosolic protein. Isoelectric focusing (IEF) was then run under several conditions using a well-established protocol[21]. The conditions were listed in Table 1. Upon completion, strips were incubated in equilibration buffer (6M urea, 75 mM Tris-HCL, pH 8.8, 29.3% glycerol, 2% sodium dodecyl sulphate (SDS), 0.0002% bromophenol blue and 1% DTT) for 15 min and another 15 min in the same buffer containing 25% iodoacetamide instead of DTT.

Second dimension separation used the 12.5% SDS slab gels at 15°C using ETTAN DALT II electrophoresis system (GE Healthcare Bioscience, Uppsala, Sweden), with the IPG strips sealed on the top surface of the gels with 0.5% agarose. The gels were subjected to electrophoresis at a constant power of 1 W/gel for 1 hr followed by 13 W/gel until the bromophenol blue marker reached approximately 1 inch from the bottom of the gel.

A 2D gel was made for each sample. Since there are 6 liver samples for each of the control and TRF-treated groups, a total of 12 gels were made. For gel visualization, Coomassie Brilliant Blue staining procedure was deployed and gels were scanned using an Imaging Densitometer (BioRad, Hertfordshire, UK). Images produced were stored in TIFF format. The 2DE map was analyzed using the Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC). Selection of spots of interest was based on the change of the spots. Spots with more than 2.0-fold change consistently were deemed as spots of interest.

## RESULTS

The proteome profile acquired from the analysis was highly reproducible between different runs and different sample sets. Representative gels were shown in Fig. 1(a, b). A sum of ~300 individual protein spots were identified with Coomassie Brilliant Blue staining. Succeeding image analysis, a total of 30 spots were detected as being differentially expressed following TRF treatment. Out of the 30 spots, only 12 individual protein spots were consistently expressed with more than 2-fold change . Thus, they were selected as spots of interest in this study. The protein spots of interest were shown in Fig. 2. The expanded montages of protein spots of interest were shown in Fig. 3.

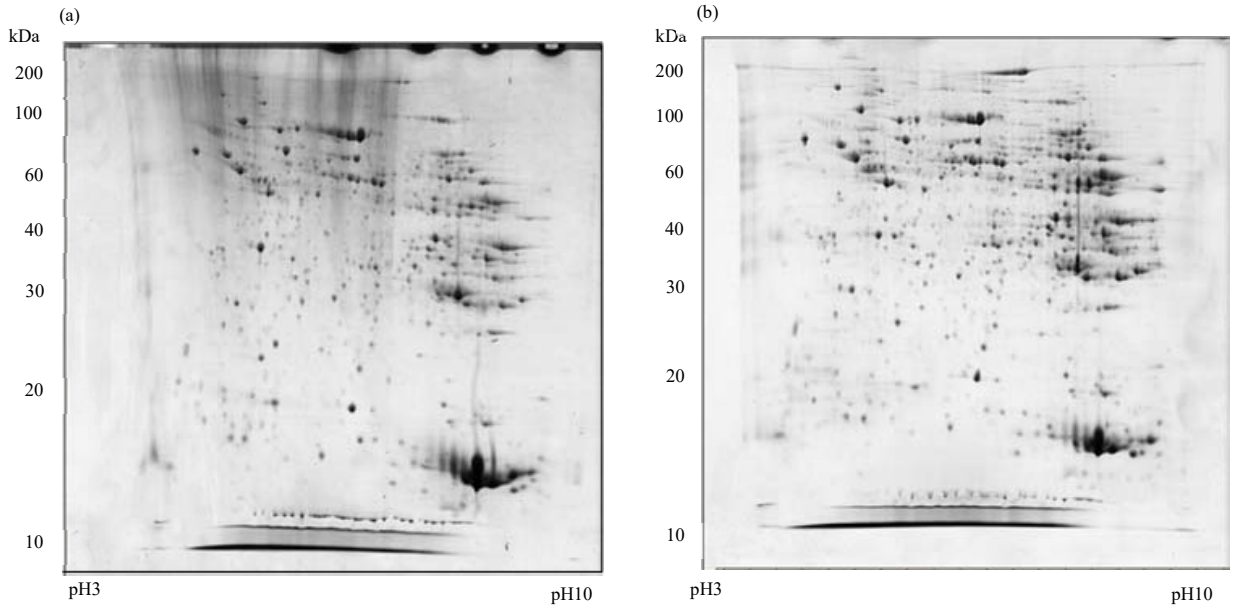


Fig. 1(a-b): Representative 2D gel images of mouse liver in the (a) Control and (b) 500 mg $kg^{-1}$ TRF treatment groups

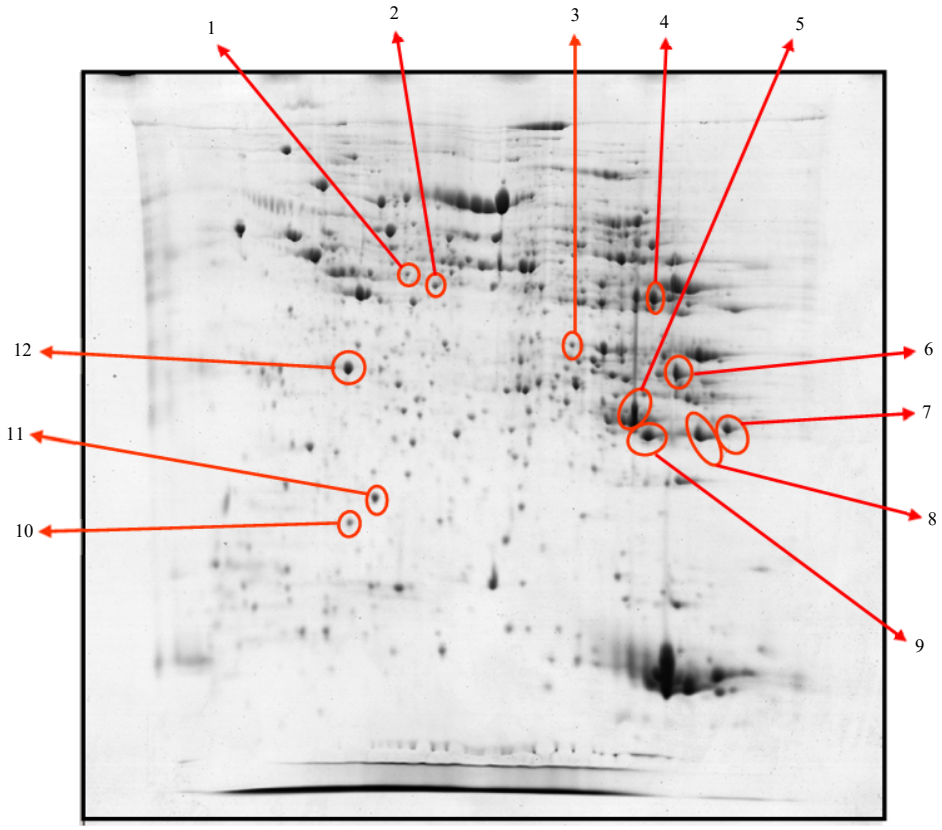


Fig. 2: Annotated 2DE map of mouse liver and the numbers indicate "protein spots of interest" in this study

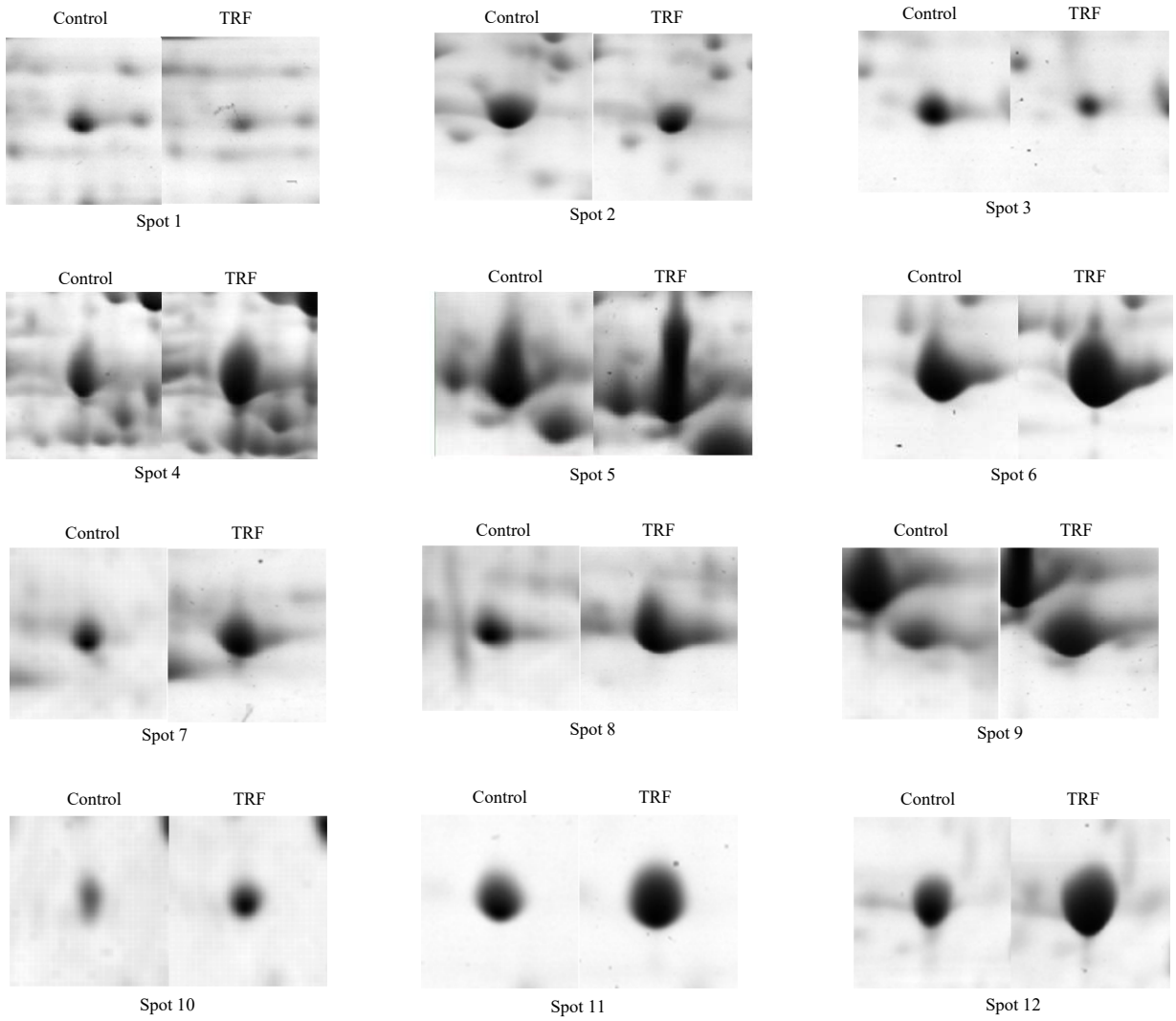


Fig. 3: Expanded montages of protein spots of interest

## DISCUSSION

In general, the 2-DE gels were vastly reproducible in terms of spot number, spot intensity and general protein profile. There were minimal intra and inter-sample variations among the 2-DE gels. Through standardized procedures of sample preparations of cells cultured, conditions for first and second dimensions of electrophoresis, gel staining and image acquisition, consistency and reproducibility were achieved. A total of 12 differentially expressed protein spots were identified and seen after treatment of mice liver cells with TRF (Fig. 2). Among the protein spots, 5 of them (spots no 1, 2, 10, 11 and 12) are acidic (pH<7) proteins. Among these five, protein spot numbered 1 has the largest molecular weight (>70 kDa), while protein spot numbered 10 is the smallest sized protein with a molecular weight of around 20 kDa. The rest (spots no 2, 11 and 12) are medium-sized proteins with a molecular weight ranging from 20 to 60 kDa. Among the protein spots, 7 of them (spots no 3, 4, 5, 6, 7, 8 and 9) are basic (pH>7) proteins. These spots were medium-sized proteins with molecular weight ranging around 30 to 50 kDA.

The TRF also exhibits different effects on different proteins (Fig. 3). It downregulated certain proteins (spots no. 1-3) and simultaneously upregulated the other proteins (spots no. 4-12). These observations indicate that TRF significantly affects several proteins and ultimately regulates batteries of molecular pathways simultaneously. Previous studies in our lab have demonstrated TRF to exhibit influence over several NRF2 proteins which are responsible for the regulation of antioxidant genes e.g., glutathione S-transferase and NAD(P)H

quinone oxidoreductase 1 in mice liver[18,20,22]. Another study by our lab revealed upregulation of liver heme oxygenase-1 expression in mice liver following treatment with TRF[19]. A recent study by our group showed that TRF activated Nrf2 nuclear translocation and increased the antioxidant-related hepatoprotective mechanism in mice liver[22]. As for this current study, there might be some association between the unknown proteins and with chemopreventive effect of TRF in mice liver. Therefore, further identification of these protein spots with more sophisticated methods such as mass-spectrometry is warranted to properly identify those proteins and provide a better understanding of the mechanism of the hepatoprotective effect of TRF.

## CONCLUSION

Our results demonstrated that TRF treatment caused specific and significant changes in the 2-DE profile of the mouse liver. The differentially-expressed protein spots that we identified might be associated with the antioxidant-related hepatoprotective mechanism in mice liver regulated by specific proteins. Further identification of these proteins by mass spectrometry may lead to a greater understanding of the mechanisms involved in the antioxidant and cytoprotective effects of TRF.

## SIGNIFICANCE STATEMENT

Tocotrienols are isomers of vitamin E which have greater antioxidant and biological activities than the more prevalent vitamin E isomers i.e., the tocopherols. There is very little attempt to study the effects of tocotrienols on liver protein expression using the proteomics approach. This study was performed to discover whether the administration of a high dose of tocotrienols (500 mg $kg^{-1}$ TRF) in mice was able to upregulate or downregulate the expression of liver proteins using the 2D gel proteomics method. Since tocotrienols have antioxidant properties, it is expected that the proteins whose expression are altered by TRF administration most likely regulates the cytoprotective aspects and redox balance of the liver.

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