Article

Protective Effects of *Tinospora cordifolia* on Hepatic and Gastrointestinal Toxicity Induced by Chronic and Moderate Alcoholism

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Abstract

Aims: Heavy alcohol intake depletes the plasma vitamins due to hepatotoxicity and decreased intestinal absorption. However, moderate alcohol intake is often thought to be healthy. Therefore, effects of chronic moderate alcohol intake on liver and intestine were studied using urinary vitamin levels. Furthermore, effects of *Tinospora cordifolia* water extract (TCE) (hepatoprotective) on vitamin excretion and intestinal absorption were also studied.

Methods: In the study, asymptomatic moderate alcoholics (*n* = 12) without chronic liver disease and healthy volunteers (*n* = 14) of mean age 39 ± 2.2 (mean ± SD) were selected and divided into three groups. TCE treatment was performed for 14 days. The blood and urine samples were collected on Day 0 and 14 after treatment with TCE and analyzed.

Results: In alcoholics samples, a significant increase in the levels of gamma-glutamyl transferase, aspartate transaminase, alanine transaminase, Triglyceride, Cholesterol, HDL and LDL (*P* < 0.05) was observed but their level get downregulated after TCE intervention. Multivariate analysis of metabolites without missing values showed an increased excretion of 7-dehydrocholesterol, orotic acid, pyridoxine, lipoamide and niacin and TCE intervention depleted their levels (*P* < 0.05). In contrast, excretion of biotin, xanthine, vitamin D2 and 2-O-p-coumaroyltartronic acid (CA, an internal marker of intestinal absorption) were observed to be decreased in alcoholic samples; however, TCE intervention restored the CA and biotin levels. Vitamin metabolism biomarkers, i.e. homocysteine and xanthurenic acid, were also normalized after TCE intervention.

Conclusion: Overall data depict that moderate alcohol intake is also hepatotoxic and decreases intestinal absorption. However, TCE treatment effectively increased the intestinal absorption and retaining power of liver that regulated alcohol-induced multivitamin deficiency.

INTRODUCTION

Chronic and moderate to heavy alcohol consumption disrupt vitamins, hormones and neurotransmitters metabolism (Rehm et al., 2009). However, these effects of alcohol vary with age, diet, gender and human race (Lang and Korzick, 2014; Butler et al., 2014; Miguez-Burbano et al., 2014). Therefore, it is difficult to understand mechanism of alcohol and it is still elusive. The first and important factor is that vitamin status in alcoholics differs from non-alcoholics. The lower levels of blood vitamins have been reported in alcoholic than healthy men (Cravo et al., 1996). It is because of the alcohol-induced inflammation that damages Kupffer cells and leads to hepatocytes necrosis (Hoyumpa, 1986; Rocco et al., 2014). Reports have suggested that low level of thiamine and folate in chronic alcoholics were due to inhibition of carrier-mediated thiamine and folate uptake in the intestine and kidney (Zhao et al., 2002; Said, 2004). Chronic alcohol consumption inhibits the conversion of pantothenic acid to Co-enzyme A. Therefore, there is an increase in the levels of free pantothenic acid, an important marker...
of PPARα inactivation (Wan et al., 1994; Nan et al., 2014). Inactivation of PPARα impairs lipid transportation and cholesterol homeostasis and leads to steatosis (Moran-Salvador et al., 2011).

A number of studies have reported the effect of alcohol intake on individual vitamins as given above. However, a few studies have been conducted which describe levels of maximum number of vitamins in biological fluids of alcoholics. Vitamins have their specific transport systems in the intestine and nephrons. Even though, all the vitamins won’t be released from the liver under influence of alcohol. Therefore, the effect of alcohol intake should vary on the vitamin absorption, re-absorption and release from the liver. This fact is supported by the findings that bio-tin has not been released from the liver after alcohol intake. Therefore, there is a need to study levels of maximum number of vitamins at the same time in the biological fluids to understand the effects of alcohol consumption on the vitamin metabolism.

Consequently, it is difficult to treat alcohol-induced disorders, and available drugs in the market are inadequate and have several side effects (Zindel and Kranzler, 2014). Furthermore, drugs available will not work for all people because of the heterogeneity of alcohol dependence (Litten et al., 2014). Therefore, inadequate efficacies of current drugs urge to develop new drugs and strategies to treat alcoholism. Kudzu (Pueraria lobata) and St. John’s Wort (Hypericum perforatum) are the excellent examples of alternate medicine, which reduce alcohol intake in alcohol-prefering rats (Perfumi et al., 1999). Tinospora cordifolia (Wild) Mier. is a herb of Menisperma-ceae family and extensively used to treat gastrointestinal problems, neuropathies, hyperlipidemia, diabetes and other disorders (Prince et al., 1998; Stanely et al., 2000; Biswadev et al., 2002; Stanely and Venugopal, 2003; Singh et al., 2003). It is known as ‘rejuvenate herb’, and Ayurveda describes it as Amrita (a drug for immortality). Therefore, in this study, we examined the effect of T. cordifolia water extract (TCE) on liver function, lipid profile and urinary vitamin levels in moderate alcoholics.

MATERIALS AND METHODS

Chemicals

Kits for biochemical assays and liver function tests were purchased from Randox Pvt Ltd (Merck Biosciences). Water, acetonitrile, methanol and formic acid, standards of fat- and water-soluble vitamin mixtures and all of mass spectrometry grades were purchased from Sigma (St. Louis, MO). Calibrants and standards for high-performance liquid chromatography-quadruple time of flight mass spectrophotometer (HPLC-Q-TOF-MS) were purchased from Agilent Technologies.

Drug preparation

Water extract (TCE) was prepared from fresh stems of T. cordifolia cultivar collected from a single host from garden of National Research Institute of Basic Ayurvedic Sciences, Nehru Garden, Kothrud, Pune, India, in September 2011. The voucher specimen (No. 207) was deposited in the medicinal plant museum of the institute. The stems were washed with deionized water, chopped and ground. The ground paste was extracted overnight with deionized water in 20:100 ratio (wt: vol) at room temperature under septic conditions. After 14 h extract was filtered. It was centrifuged at 12,000 rpm, and a fresh extract (3.0 g after lyophilization) was given orally to the volunteers to avoid degradation of phyto-constituents, previously reported from our lab (Shiralkar et al., 2013). Random extracts were subjected to LC-MS and analyzed to find out the difference in the chemical constituents of stems, if any.

Study subjects

Initially, volunteers were selected on the basis of drinking status, gender and age. All the volunteers underwent the questionnaire of alcohol use disorders identification test (AUDIT) for assessment of alcohol abuse. Alcohol is reported to have different effects on the genders and age groups. Therefore, in this study, only males of mean age 39 ± 2 (mean ± SD) having a body weight between 62.3 ± 2.2 (mean ± SD) were selected out of 230 volunteers. As duration and dose amount of alcohol intake also have different effects, only the volunteers regularly drinking 60–80 ml of 80 proof liquor (40% alcohol) (24–36 g) per day from the last one year and at least 5 days in a week were included in the study. Chronic moderate alcoholic (n = 12) and non-drinking healthy males (n = 14) who completed the above criteria were selected for the study from the local areas of Pune, Maharashtra, India. Male volunteers having a mass index >30 kg/m², blood pressure >160/90 mm/Hg, total cholesterol >7.5 mmol/l, present or prior history of cardiovascular disease, respiratory, renal, gastrointestinal, hepatic, endocrine, diabetes mellitus or reproductive disorders; or use of lipid-lowering or anti-hyper-tensive agents were excluded from the study. Human Ethics Committee of the DYP Ayurveda College, Pune, India, approved the study wide letter no. RRI/2011/HEC/2023 dated 18-11-2011. All the volunteers have signed an informed written consent.

Study design and dietary intervention

Volunteers have attended the day care center and served breakfast, lunch and dinner. All the participants were watched for 1 week, called the pre-watch period before medication, and patterns of liver enzyme were recorded. Afterward, all the healthy and alcoholic volunteers were given freshly prepared 100 ml TCE (3.0 g solid extract) early in the morning with an empty stomach for 14 days in the presence of an Ayurveda physician. The complete work flow of the study design is shown in Figure 1. Vegetarian diet containing around 9000 kJ of energy was served to all the volunteers after the dose of TCE. It basically includes one cup tea or one cup low fat milk, one small bowl of cornflakes or two slices of bread with teaspoon of butter, one cup baked beans in the morning breakfast after TCE dose. Lunch included rice, pulses and chapattis (Indian style breads of wheat flour) along with salad and curd. Vegetables, chapattis and salad were served in dinner. The alcoholics continued ~60 ml of 80 proof liquor (40% alcohol) consumption per day (0.5 g/kg/body weight corresponding to three standard drinks per day) during the treatment period of 14 days. First pass urine and fasting blood samples were collected on 0 and 14th day, and urine samples were treated with 2.5 mM sodium azide. Urine samples were centrifuged, filtered (0.2 µm filters) and stored at ~80°C till analysis.

Liquid chromatography-coupled with mass spectrometry

ZORBAX 300SB C-18 (3 x 100 mm, 2.7 µm particle size) with a guard column fitted Agilent 1290 Infinity Series HPLC interfaced to Agilent 6538 Accurate-Mass Q-TOF-MS was used to resolve unprocessed urine samples. Urine samples were diluted to 2-fold with acetonitrile–water (80:20, vol:vol), centrifuged at 8000 rpm for 5 min and 200 µl of samples were injected into the column by an auto-injector. The mobile phase had 0.1% formic acid in (A) water and (B) aceto-nitrile. Mobile phase A was set up to increase from 5 to 95% in a linear gradient mode for initial 40 min and then decreased to 5% up to 45 min and remained constant till 50 min. A constant flow rate of 0.2 ml/min of the mobile phase was used. Q-TOF-MS was operated in a positive ion polarity mode, with extended dynamic range.
(1200 m/z, 2 GHz), ramped collision energy, fragmentor voltage 195 V and 3 spectra/s acquisition rate. The machine was operated with continuous internal calibration during data collection.

**Quality assurance**

Quality of data was assured using three different steps. First, the mixtures of vitamins were injected to check the operational conditions of the HPLC-Q-TOF-MS before the sample analysis, and Personal Compounds Database Library (PCDL) having accurate retention time and the product ions was prepared. Second, urine samples were spiked with the vitamins (2.5 ppm) to check the peak volumes and intensity with non-spiked samples. Finally, quality control of urine samples was performed by mixing the urine samples of specific groups so that the actual levels of vitamins in healthy, alcoholics and alcoholics treated with TCE can be compared with the individual of that group.

**Selected metabolite identification**

MassHunter (Qualitative Analysis Version B.04.00 from Agilent Technologies) software was used to process raw mass data. A small library of MS–MS spectra of standard vitamins was prepared. Spectra of vitamins and other compounds were extracted from raw data using prepared PCDL and keeping minimum ion abundance to 5000 cups.
Vitamins were identified by comparing the exact mass, fragmentation pattern and retention time with a confidence level of 97.98%, mass error >5.0 ppm and retention time variation >0.2 min. Datasets were imported into Mass Profiler Profession (MPP) software (Version. B.02.02; Agilent Technologies) and aligned using the retention time window and mass difference of 0.2 min and 5.0 ppm, respectively. Vitamins that were present in <75% of the samples of a group and having \( p < 0.05 \), fold change <2.0 and CV >15 were removed from datasets and further subjected to principal component analysis. Final data were subjected to the Wilcoxon paired t-test with one-way ANOVA and Benjamini–Hochberg multiple testing correction, keeping up value cut off <0.05 and fold change ≤2.0.

Statistics

Gamma-glutamyl transferase (\( \gamma \)-GT), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and mean corpuscular volume (MCV) values are expressed as the mean ± SD. During the treatment period, no major interactions were identified that permitted to analyze alcohol intakes and alcohol intakes plus TCE treatment periods for comparison by the paired t-test.

RESULTS

Characterization of TCE

A very simple solvent system and scheme was used to resolve TCE components. Compounds present in TCE were resolved over a C-18 ZORBAX Eclipse Plus column (C18, 4.5 x 250 mm) of 5 \( \mu \)m particle size. Mobile phase comprised Solvent A (water containing 0.1% formic acid) and Solvent B (acetonitrile containing 0.1% formic acid) were used in gradient mode [min/%] for Solvent B 5%/8; 20%/15; 90%/35; 5%/40 with 0.5 ml/min flow rate. Q-TOF-MS was used with optimized parameters as described earlier (Shirokar et al., 2013; Mittal and Dabur, 2015). The total ion chromatogram and mass spectrum of TCE showed highest abundance of magnoflorine (product ions \( m/z \) 237.09, 265.09, 297.12) with other known compounds. Unknown O-glucoside of \( m/z \) 345 (product ion \( m/z \) 365), 775.6 (product ions \( m/z \) 519.31, 497.32, 184.08), 413.39 (product ions \( m/z \) 109.06, 123.08, 217.16, 271.16, 396.38), 383.16 (product ions \( m/z \) 269.12, 365.12), 297.12 (product ions \( m/z \) 153.07, 207.08, 219.08, 237.09, 282.09), 274.28, 174.15, 160.13 and 104.10 were also observed. Various minor compounds including columbin, citramalic acid, tinocordifoliside and myricetin were also present across the entire sample (Table 1). Multivariate analysis showed consistency in metabolomic profiles of TCE samples, and the correlation coefficient of chromatograms was within 0.987 ± 0.021 (mean ± SD). Freshly prepared TCE has brown color, with 7.57 pH, having slightly bitter taste and no characteristic odor. It has a specific gravity of 1.1651 and a viscosity of 1.5534 cP.

Blood biochemistry

\( \gamma \)GT levels in alcoholsics were consistent on Day 0 and 14 showing the persistent alcohol intake. In alcoholsics, its levels were found to be increased to 40.3 ± 2.3 and 38.9 ± 2.7 U/I on Day 0 and 14, respectively; whereas in healthy individuals, it was found to be 22.6 ± 3.1 U/I. TCE treatment depleted the level to 28.4 ± 2.2 U/I, \( p < 0.01 \). The AST/ALT ratio was found to be 1.09 in healthy individuals, which increased to 1.37 in alcoholsics and decreased to 1.15 in TCE-treated individuals (Table 2). The ALP level was found to be increased significantly in alcoholsics and depleted by TCE treatment when observed on Day 14 (mean ± SD; 261.5 ± 2.1 U/I in alcoholsics to 95.5 ± 2.1 U/I in alcoholsics treated with TCE; \( P < 0.001 \)). However, ALP levels were still higher even after treatment, which may be because of continuous alcohol consumption during the treatment period. Uric acid levels were reduced in alcoholsics (mean ± SD; 6.9 ± 0.3 to 4.3 ± 0.5 mg/dl on Day 0, \( P < 0.05 \)) and while TCE treatment increased its levels on Day 14 to 5.1 ± 0.4 mg/dl. MCV was recorded to be significantly increased in alcoholsics (mean ± SD; 86.3 ± 0.5 fl in healthy individuals to 96.7 ± 0.7 fl in alcoholsics on Day 0 and 87.9 ± 0.5% in alcoholsics treated with TCE on Day 14, \( P < 0.01 \)) (Table 2). Triglyceride (TGL), Cholesterol (CHL) and LDL levels were observed to be increased to 191.1 ± 9.1, 247.3 ± 11.1 and 240.5 ± 6.5 mg/dl, respectively, in alcoholsics. However, TCE treatment decreased the levels of TGL, CHL and LDL to 136.2 ± 9.2, 181.7 ± 9.2 and 182.3 ± 5.5 mg/dl, respectively (\( P < 0.05 \)). Alcohol consumption also increased the HDL level to 81.1 ± 7.5 mg/dl as compared with healthy men (60.0 ± 4.2 mg/dl), and TCE did not found to affect its levels much. Na, K and Ca\(^{2+}\) excretion levels in urine seem to be less affected by moderate alcohol consumption; however, slightly increased levels of Na and K and decreased levels of Ca\(^{2+}\) were observed (Table 2).

| Table 1. The list of predicted metabolites in TCE along with their mass and retention time |
|-----------------|-----------------|-----------------|
| S. no. | Tentative compounds identified | Mass | Retention time |
| 1 | Tinocordifolioside | 412.12 | 29.94 |
| 2 | N-Methyltetrahydrcolumbamine | 355.30 | 42.05 |
| 3 | 5,8,13,13a-Tetrahydrocolumbamine | 341.17 | 26.44 |
| 4 | Tetrahydropalmitamine | 355.19 | 27.41 |
| 5 | Dihydroberberine | 337.14 | 28.82 |
| 6 | Triterpenoid glucoside | 484.37 | 25.08 |
| 7 | 8-Hydroxycolubinin | 374.15 | 29.87 |
| 8 | Myricetin | 315.21 | 25.86 |
| 9 | 24-Epi-Makisterone A | 494.34 | 26.34 |
| 10 | Isolalicresinol 9-O-β-D-glucoside | 521.22 | 23.94 |
| 11 | Palmatine | 352.23 | 30.30 |
| 12 | Dihydoceramide | 329.34 | 41.03 |
| 13 | Magnoflorine | 342.16 | 28.25 |
| 14 | Myristicin | 192.97 | 7.08 |
| 15 | Tinosporaside | 500.17 | 18.34 |
| 16 | 20-Deoxymakisterone A | 480.32 | 25.97 |
| 17 | Dideoxyecdysone | 443.30 | 25.97 |
| 18 | Columbin | 358.38 | 29.81 |
| 19 | Unknown compound | 544.01 | 28.44 |
| 20 | Unknown compound | 774.61 | 41.13 |
| 21 | Unknown compound | 412.39 | 26.16 |
| 22 | Unknown compound | 382.16 | 31.78 |
| 23 | Unknown compound | 296.12 | 27.26 |
| 24 | Unknown compound | 273.28 | 34.79 |
| 25 | Unknown compound | 173.15 | 15.56 |
| 26 | Unknown compound | 159.13 | 9.7 |

Water-soluble vitamin levels by conventional methods

Analysis showed that vitamins can be categorized into two categories; one with decreased excretion and other with increased excretion. Riboflavin levels were depleted in alcoholsics from 3.71 ± 1.07 to 2.12 ± 0.5 µmol/day on Day 0 and increased after TCE intervention to 2.13 ± 0.4 µmol/day on Day 14 (\( P < 0.05 \)). Biotin levels were found to be decreased from 43 ± 7.1 to 23 ± 9.1 µmol/day, which increased after TCE intervention to 39 ± 5.5 µmol/day. Levels of pantothenic acid were found to be decreased in alcoholsics from 23.1 ± 2.3 to 15.3 ± 3.0 µmol/day. However, TCE treatment increases...
it to 27.2 ± 1.6 µmol/day. In terms of fold change, riboflavin, biotin and pantothenic acid excretion in TCE-treated alcoholics were found to be 1.50, −2.63 and 1.42 folds, respectively (Tables 3 and 4). Urinary levels of thiamine increased from 2.43 ± 0.8 to 4.27 ± 0.5 µmol/day on Day 0 and found to be decreased after TCE treatment to 3.11 ± 0.14 µmol/day on Day 14 (P < 0.05). Niacin excretion was observed to increase from 75 ± 7.1 to 321 ± 17.3 µmol/day in alcoholics, which decreased after TCE treatment to 119 ± 9.9 µmol/day (P < 0.05). TCE intervention also significantly decreased the urinary excretion of pyridoxine (19.3 ± 1.7 to 8.3 ± 0.8 µmol/day), folate (87 ± 9.3 to 39 ± 3.3 nmol/day), cobalamin (156 ± 11.3 to 114 ± 8.4 µmol/day) and ascorbic acid (297 ± 11.7 to 145 ± 7.3 µmol/day) on Day 14. In terms of fold change, thiamine, niacin, pantothenic acid, pyridoxine, folate, cobalamin and ascorbic acid levels were decreased in urine of alcoholics after TCE intervention from 2.37 to 1.05, 2.29 to 1.24, −2.16 to −1.42, 2.04 to 1.30, 2.26 to 1.18, 1.61 to 1.17 and 1.16 to 1.57 folds, respectively (Table 4).

### Table 2. Blood biochemistry of healthy, chronic moderate alcoholics and alcoholics treated with TCE on Day 0 and 14 (means ± standard error)

<table>
<thead>
<tr>
<th>Test</th>
<th>Healthy Day 0 (n = 14)</th>
<th>Alcoholic Day 0 (n = 12)</th>
<th>Alcoholic Day 14 (n = 12)</th>
<th>TCE treated Day 14 (n = 12)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>y-GT (U/l)</td>
<td>22.6 ± 3.1</td>
<td>40.3 ± 2.3</td>
<td>38.9 ± 2.7</td>
<td>28.4 ± 2.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>21.5 ± 0.3</td>
<td>53.3 ± 0.7</td>
<td>51.1 ± 0.7</td>
<td>25.7 ± 0.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>19.7 ± 0.5</td>
<td>38.9 ± 0.5</td>
<td>37.4 ± 0.5</td>
<td>22.3 ± 1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST/ALT</td>
<td>1.09</td>
<td>1.37</td>
<td>1.36</td>
<td>1.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>86.3 ± 0.5</td>
<td>96.7 ± 0.7</td>
<td>94.8 ± 0.4</td>
<td>87.9 ± 0.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>69.5 ± 1.7</td>
<td>271.1 ± 2.3</td>
<td>261.5 ± 2.1</td>
<td>95.5 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>6.9 ± 0.3</td>
<td>4.3 ± 0.5</td>
<td>4.5 ± 0.5</td>
<td>5.1 ± 0.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hemoglobin (%) (%/dl)</td>
<td>16.2 ± 0.3</td>
<td>13.4 ± 0.1</td>
<td>13.6 ± 0.2</td>
<td>15.2 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TGL (mg/dl)</td>
<td>116.3 ± 10.3</td>
<td>191.1 ± 9.1</td>
<td>179.6 ± 8.2</td>
<td>136.2 ± 9.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>169.1 ± 7.3</td>
<td>247.3 ± 11.1</td>
<td>233.3 ± 7.1</td>
<td>181.7 ± 9.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>60.0 ± 4.2</td>
<td>81.1 ± 7.5</td>
<td>75.2 ± 3.1</td>
<td>76.3 ± 4.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Na (mmol/l)</td>
<td>134.9 ± 2.9</td>
<td>137.6 ± 2.7</td>
<td>138.5 ± 3.1</td>
<td>135.3 ± 2.5</td>
<td>–</td>
</tr>
<tr>
<td>K (mmol/l)</td>
<td>4.28 ± 0.2</td>
<td>4.79 ± 0.2</td>
<td>4.77 ± 0.16</td>
<td>4.34 ± 0.11</td>
<td>–</td>
</tr>
<tr>
<td>tCa (mmol/l)</td>
<td>2.93 ± 0.1</td>
<td>2.83 ± 0.2</td>
<td>3.11 ± 0.14</td>
<td>2.99 ± 0.14</td>
<td>–</td>
</tr>
</tbody>
</table>

*P = Number of volunteers enrolled in the study.  
*P values were calculated by using microCal origin software by utilizing one-way ANOVA analysis.

### Table 3. Levels of urinary water-soluble vitamins in chronic and moderate alcoholic males on the controlled diet

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Control</th>
<th>Alcohols Day 0</th>
<th>Alcohols Day 14</th>
<th>TCE Treated ALC Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>2.43 ± 0.8 µmol/day</td>
<td>4.27 ± 0.5 µmol/day</td>
<td>4.13 ± 0.3 µmol/day</td>
<td>3.11 ± 0.3 µmol/day</td>
</tr>
<tr>
<td>Biotin</td>
<td>43 ± 7.1 µmol/day</td>
<td>23 ± 9.1 µmol/day</td>
<td>20 ± 11.3 µmol/day</td>
<td>39 ± 5.5 µmol/day</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>3.71 ± 1.0 µmol/day</td>
<td>2.12 ± 0.5 µmol/day</td>
<td>2.13 ± 0.4 µmol/day</td>
<td>3.11 ± 0.3 µmol/day</td>
</tr>
<tr>
<td>Cobalamin</td>
<td>97 ± 7.3 µmol/day</td>
<td>156 ± 11.3 µmol/day</td>
<td>144 ± 8.4 µmol/day</td>
<td>114 ± 8.7 µmol/day</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>23.1 ± 2.3 µmol/day</td>
<td>15.3 ± 3.0 µmol/day</td>
<td>15.2 ± 1.7 µmol/day</td>
<td>27.2 ± 1.6 µmol/day</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>121 ± 8.3 µmol/day</td>
<td>297 ± 11.7 µmol/day</td>
<td>265 ± 13.1 µmol/day</td>
<td>145 ± 7.3 µmol/day</td>
</tr>
<tr>
<td>Folic acid</td>
<td>33 ± 2.1 nmol/day</td>
<td>87 ± 9.3 nmol/day</td>
<td>73 ± 5.3 nmol/day</td>
<td>39 ± 3.3 nmol/day</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>5.0 ± 1.1 µmol/day</td>
<td>19.3 ± 1.7 µmol/day</td>
<td>16.6 ± 2.3 µmol/day</td>
<td>8.3 ± 0.8 µmol/day</td>
</tr>
<tr>
<td>Niacin</td>
<td>75 ± 7.1 µmol/day</td>
<td>321 ± 17.3 µmol/day</td>
<td>301 ± 11.2 µmol/day</td>
<td>119 ± 9.9 µmol/day</td>
</tr>
</tbody>
</table>

All values are expressed in mean ± SD.

N1-methylisonicotinamide, N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-3-carboxamide concentrations were included with niacin. 4-Pyridoxic acid concentration was included with pyridoxine. The significance value by the Student t-test was found to be P < 0.05 for all the tested vitamins when comparisons made in between the alcoholics to TCE treated or control.

### Multivariate analysis

Normalized ion abundance data of vitamins and selected compounds after retention time and m/z alignment were compared using a Whisker Box plot. The box-plot analysis showed the different patterns of metabolites among healthy, alcoholics and alcoholic treated with TCE groups (Figure 2A). Furthermore, data were filtered using the strategies discussed in ‘Materials and methods’ section and metabolites having significance P > 0.05 and CV < 15 were filtered out. Careful analysis of data of individual samples showed the missing values of the metabolites and vitamins. Therefore, to maintain the consistency of data and 100% confidence, the metabolites with missing values were filtered out. A Spearman correlation heat map is shown in Figure 2B. The heat map constructed from the data showed only 12 metabolites in which only biotin and 2-O-p-coumaroylshartronic acid were found to be decreased (Figure 2B). Scattered plots of data showed that alcoholics treated with TCE were found to move toward the healthy group, showing the recovery of the alcoholic group and
effectiveness of the TCE intervention (Figure 3). Reversal in positions of xanthine, xanthurenic acid, nicotinic acid, vitamin D2, cystathionine, liposamide, pyridoxine, and orotic acid were found to be increased throughout the samples. Therefore, these are the most affected metabolites in alcoholics. Further, the color patterns in the heat map of other metabolites including vitamin D2, xanthine, xanthurenic acid, liposamide and cystathionine show TCE-treated alcohols coming near to the healthy individuals group while the alcoholic group lies far apart (Figure 4B). The heat map of the metabolites without missing values was implemented to identify and rank signature metabolites explaining most of the variance between the three groups.

Effect of TCE treatment on urinary excretion of vitamins
Multivariate analysis showed differential expression of 24 compounds including 9 vitamins. Identification of vitamins was further confirmed by matching mass fragments and retention time with the standards. Biotin gave product ions at m/z 227 (−18 Da), 209, 199, 166 and 123. Excretory biotin levels in alcohols were found to be depleted by −2.22-fold (Figure 4B and B1). TCE treatment did not show any significant alteration in the biotin levels. Riboflavin found to be decreased in alcohols by −3.71-fold and increased to −1.50-fold after TCE treatment. It elutes at 18.21 min and generates a strong ion peak at m/z 377. Further cleavage produces ions at m/z 359 due to loss of water and m/z 243 due to loss of ribitol chain. Pantothentic acid elutes at 9.26 min. It (M+H)+ produces two product ions at m/z 202 and 184 due to successive loss of water molecules. Cα-CO bond cleavage resulted in an acyl ion at m/z 116. Pantothentic acid was found to be decreased in alcohols by −2.16-fold, which increased to −1.42-fold in alcohols after 14 day treatment with TCE. The urinary liposamide level was found to be increased by 1.63-fold in alcohols that get depleted after TCE treatment to 2.68-fold (Figure 4C and C1) (Table 4). Table 5 describes the detected vitamins without missing entries and their normalized signed fold change after the Wilcoxon paired t-test.

TCE treatment brought down the thiamine concentration in urine to the normal concentration, i.e. 2.37-fold to −1.05-fold. Thiamine elutes at 16.1 min and generates two product ions at m/z 122 (pyrimidinic ring) and 144 (thazolic ring). Niacin levels also reduced in alcohols after TCE intervention from 2.29- to 1.24-fold (Figure 4D and D1). Niacin was identified for its retention time 14.31 min and isomeric mass. A minor increase in the levels of adenine (vitamin B4) (1.4-fold) was observed in alcohols, which decrease to −1.6-fold after TCE intervention. Adenine was eluted at 6.56 and identified for its specific fragments at m/z 119 and 107 due to loss of ammonia and breaking of the ring. Pyridoxine (vitamin B6) elutes at 11.6 min and gives product ions at m/z 152 and 134 due to successive loss of water. Specific product ions for pyridoxine were found at m/z 124 and 111. Urinary pyridoxine levels were observed to be increased by 2.04-fold in alcohols that get depleted after TCE treatment to 1.30-fold (Figure 4E and E1). Other than vitamin B, vitamin D2 was observed to be decreased in alcohols by −1.82-fold, which increase after TCE intervention to 1.45-fold. Vitamin D2 was eluted at 47.6 min and identified using fragments at m/z 107, 121, 133, 147, 201, 271, 309 and 379 (Table 3).

Other metabolites searched and found decreased in the urine samples of alcohols and increased after TCE treatment were γ-glutamyl ornithine (found only in healthy and TCE treated), phenylacetylglycine (detected only in alcohols), tryptophan (−1.30-fold in alcohols and 1.24-fold in TCE-treated alcohols) and xanthurenic acid (1.99-fold in alcohols and −2.0 in TCE-treated alcohols) (Figure 4F and F1) when compared with counterparts on control-diet. Some metabolites, i.e. homocysteine and carnitine, were found to be increased in the urine samples of alcohols by 2.43- and 1.52-fold and depleted significantly after TCE intervention to −2.10- and −1.61-fold, respectively.

DISCUSSION
Heavy alcohol consumption dilutes the micronutrients including vitamins. In contrast, light-to-moderate alcoholism is described to be healthy. However, studies on prolonged moderate alcohol intake in controlled environments are scanty. Therefore, in this study, we use 7 days as the wash out period and to stabilize the conditions. Initial biochemistry analysis showed good consistency in the conventional biomarkers of alcoholism, i.e. γGT and MCV (Table 2). In the study, conventional as well as Q-TOF-MS analysis showed almost the same results except minor variation (Tables 3 and 4). Q-TOF-MS as well as conventional analysis showed increased urinary excretion of vitamins except riboflavin, pantothenic acid and biotin.

Prolonged moderate alcohol consumption also induce the TNFα from Kupffer cells just like heavy alcoholism and induce hepatocytes injury through activation of CYP2E1 to metabolize alcohol and produce superoxide. Injured hepatocytes lose the capability to retain vitamins and release them in serum, whereas reduced absorption in nephrons increase the urinary excretion of vitamins (Sorrell et al.,

<table>
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<tr>
<th>S. no.</th>
<th>Compound</th>
<th>ALC v/s HLT</th>
<th>ALC v/s TRT</th>
<th>HLT v/s TRT</th>
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All the fold change values are normalized signed fold change.
Fig. 2. (A) Box Whisker plots of healthy (HLT), alcoholics (ALC) and alcoholics treated with TCE (TRT) of normalized data showing the distribution pattern of vitamins among the groups. Bottom and top of the boxes indicate 25th and 75th percentiles, and whiskers indicate 5th and 95th. The figure clearly represents the distinctness between the three groups and closeness in HLT and TRT groups. (B) Spearman correlation heat map alignment of the variables presents throughout the sample without any missing values, with identified metabolites marked on the side of the map and given in Table 3. Each row represents a sample and each column represents a metabolite feature. Color key indicates the normalized abundance of each metabolite expression value across the samples. The red and blue color (black and gray in print) indicates highest and lowest metabolite expression values respectively. CA represents 2-O-p-Coumaroyltartrontronic acid.

Fig. 3. Scattered plot of vitamins and other related metabolites without any missing value of healthy (HLT), alcoholics (ALC) and treated (TRT) groups with fold change lines. Figure (A) represents ALC v/s HLT and (B) represents ALC v/s TRT. Each square dot represents a single identified metabolite displaying its up-regulation or down-regulation. The figure shows metabolites with increased or decreased expression in each respective individual plot. The plot clearly indicates the altered levels of compounds in the treated group in comparison to alcoholics, particularly in xanthurenic acid, biotin, xanthine, vitamin D2, pyridoxine, niacin and lipoamide.
The study indicates that the liver lost the capability to retain pyridoxine (Figure 4F and F1), niacin (Figure 4D and D1), ascorbic acid and folic acid initially under influence of alcohol and therefore, increased excretion was observed (Table 4). Pyridoxine and niacin are the most affected vitamins, also confirmed in multivariate analysis. TCE reduced hepatotoxicity, clearly established by the reduced activities of traditional biomarkers of alcoholism, i.e. γ-GT, AST, ALT, ALP and decreased the ratio of AST/ALT from 1.37 to 1.15 (Table 2). Levels of xanthine and xanthurenic acid have been reversed after treatment with TCE, which are known biomarkers of alcoholism (Mittal and Dabur, 2015). TCE components amritosides, palmarin and tinosporide are found to be agonists of opioid receptors and help in liver regeneration as well to reduce cravings (Sharma and Pandey, 2010; Mittal and Dabur, 2015). Data showed that TCE increased the retaining capability of the liver and therefore, decreased the excretion of pyridoxine (Figure 4F and F1) and niacin (Figure 4D and D1), which was due to liver regeneration and reduced oxidative burden.

Urinary levels of biotin (Figure 4B and B1), pantothenic acid and riboflavin were decreased in alcoholics, which indicate the different retention mechanism or reduced intestinal absorption. Low biotin concentration in serum, alcohol and acetaldehyde are reported to effectively inhibit biotin absorption across the intestinal wall (Subramanya et al., 2011). Studies have reported that the intestinal biotin uptake system (sodium-dependent multivitamin transporter) also

<table>
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<tr>
<th>S. no.</th>
<th>Compound</th>
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<th>P value (Corr)</th>
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<th>FC ([TRT] vs [ALC])</th>
<th>FC ([HLT] vs [TRT])</th>
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<td>1</td>
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</tbody>
</table>

Fig. 4. Box Whisker and profile plots of 2-O-p-coumaroyltartronic acid (A, A1), biotin (B, B1), lipoamide (C, C1), niacin (D, D1), pyridoxine (E, E1) and xanthurenic acid (F, F1) showing up- and downregulation of various metabolites and vitamins after moderate alcohol intake and treatment with TCE. ALC, HLT and TRT represents alcoholic, healthy and TCE-treated alcoholics, respectively.
transports the pantothenic acid and lipoic acid (important metabolic for redox regulation). That may be one reason of pantothenic depleted levels in urine. This is supported by decreased urinary excretion of 2-O-β-coumaryloxytryptamine acid (Figure 4A and A1) and 9-O-methyl-coumeestrol by 2.2 and 3.6-fold (P < 0.05), respectively, in alcoholics, biomarkers present in food and increased their excretion after TCE intervention (Table 4). Overall, alcohol intake decreased the intestinal absorption of biotin, pantothenic acid and CA, therefore decreased their urinary excretion.

Decreased levels of cystathionine and increased levels of homocysteine have been observed in the study. Folic acid deficiency is a common complication in chronic alcoholics and acute decrease in serum (Herbert et al., 1963; Savage et al., 1994), and increased folate levels in urine of chronic alcoholics have been reported (Russell et al., 1983). Deficiency in folic acid and cobalamin leads to aberrant methionine metabolism and prevents the homocysteine to be metabolized into methionine. At the same time, deficiency in pyridoxine blocks the conversion of homocysteine to cystathionine (Halsted et al., 2002). Ultimately, it accumulates homocysteine in serum and decreased re-absorptive transport of folate by renal proximal tubule cells (Rachelle et al., 2007), resulting in its increased levels in urine. Aberrant methionine metabolism also decreases levels of S-adenosyl methionine levels, which plays an important role in histone methyla
tion and leads to liver injury due to insufficient of antioxidant defence mechanisms. Catabolism of alcohol produces superoxide and hydroxyl radicals exerting deleterious effects to the liver. The anti-oxidant activity of TCE is well documented because of the presence of phenolic compounds, ascorbic acid and its ability to induce GSH synthesis. This is supported by decreased urinary folate levels (Figure 4C and C1) after TCE treatment, known to maintain cell redox state. Columbin and triterpene-fatty acid esters in TCE have been reported for its anti-inflammatory activities through the inhibition of cycloxygenase-2 and nitric oxide (Li et al., 2004; Ibrahim et al., 2012). Further, TCE was found to be enriched in magnoflorine, palmatine and jatrorrhizine (Table 1), known to have hypolipidi
demic and anti-diabetic effects. Therefore, TCE intervention, decreased excretory levels of homocysteine, folate, pyridoxine and cobalamin in alcohols indicate that TCE increased the efficiency of the antioxidant defense mechanism. Additionally, increased hemoglobin levels in TCE-treated alcoholics indicate that the TCE intervention restored folic acid as well as cobalamin synthesis or their level. Further, depletion of carnitines in the TCE-treated alcoh
colic group supports the anti-hyperlipidemic properties of TCE (Table 3) through PPARα activation by palmatine and magnoflorine as reported earlier (Sangeetha et al., 2013). Quercetin and berberine have also been reported to activate SIRT 1 and PGC-1α through activa
tion of AMP kinase, which activates mitochondria biogenesis. These molecules also inhibit CPT1 and ACC2 resulting in increased β-fatty acid oxidation. In conclusion, all the activated processes by TCE helped to reduce oxidative stress induced by alcohol and therefore the injuries caused by it.

CONCLUSION

Controlled environmental analysis has shown that prolonged moderate alcohol consumption increases the urinary levels of production, niacin, ascorbic acid and folic acid, especially, by inhibiting hepatic storage and nephron re-absorption. Decreased levels of biotin were mainly due to intestinal absorption. However, moderate alcohol in
take has very less impact on riboflavin and pantothenic acid metabolism and their excretion. Overall, prolonged moderate alcohol intake in chronic form is not safe. TCE intervention effectively reversed the urinary excretion of vitamins, altered the excretion of metabolic intermediates of vitamins and restored normal vitamin metabolism. TCE intervention normalized vitamin metabolism by decreasing oxidative stress through antioxidant compounds present in it. Further, magnoflorine and columbin trigger the liver regeneration through dopamine receptors and PPARα activation. Moreover, TCE increased glutathione synthesis and lipoamide levels, which reduced inflammation and helped in liver regeneration that retained vitamins. TCE also increased the uptake of biotin, and CA indicated its capability to increase intestinal absorption. Overall, TCE ameliorates the ef
effect of alcohol on vitamin metabolism in alcoholic men due to its capability to fight oxidative stress, regenerating liver, increasing intesti
tinal absorption and modulation of lipid metabolism. Therefore, it may be used as a stand-alone or as an adjuvant with other therapies to treat alcohol-induced disorders.

FUNDING

The author acknowledges DST-FIST and HSCST programmes for their financial assistance for some of the research instruments.

CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

inflammatory activities of columbin through the inhibition of cycloxygenase-2 and nitric oxide but not the suppression of NF-κB translocation. Eur J Pharmacol 678:61–70.
toxicity and active compounds of Tinospora smilacina Benth. Phytoster Res 18:78–83.


