Orthosiphon stamineus: an Asian tea with substantial anticancer properties

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Abstract

Background: Liver cancer is one of the deadly cancers with high prevalence in the East Asia. Likewise most of diseases, herbs and herbal medicine could be an easy and cost effective tool in prevention and possible cancer treatment. The present study investigated the ability of Orthosiphon stamineus Benth decoction to protect liver against hepatocellular carcinoma in carcinogenesis-induced animal model.

Methods: Forty male Sprague Dawley rats (age: 8±1 weeks, weight: 248.1±7.21g) were obtained and 10 rats were kept as normal group. Hepatocellular carcinoma was induced for the rest 30 of rats by means of intraperitoneal injection of 200mg/kg diethyl nitrosamine (DEN) dissolved in corn oil. Induced cancer rats were under hepatocarcinogenesis promoter diet made from a mixture of standard rat diet (AIN-76) with 2-acetylaminofluorene (0.02% AAF) for two weeks. Two weeks after this diet, left over rats were divided to two groups as control and treatment. Treatment group, were forced feed daily with 0.7 ml O. stamineus decoction.

Results: After 28 weeks treatment with O. stamineus decoction, serum biochemical markers including alpha fetoprotein (AFP), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), corticosteroid binding globulin (CBG), gamma glutamyl transferase (GGT), homocysteine (HCY), tumor necrosis factor alpha (TNF\texttextsuperscript{-}\alpha), and alpha 2 macroglobulin (\alpha2MG) have been regulated favorably. Total antioxidant status (TAS) also has been increased drastically. Liver lesion score in treated groups were reduced and glucocorticoid activity has been amplified significantly.

Conclusion: Our results indicate that O. stamineus decoction might prevent or subdue liver cancer development.

Keywords: Cell proliferation, Glucocorticoid, Cell receptors, Phytochemicals, Caspase

Introduction

Herbal teas and their decoctions have a long history in human life, especially in East Asia. Different types of herbs are an important part of medical history and have been widely used to cure or prevent diseases. Orthosiphon stamineus Benth or Cat's Whiskers (family: Lamiaceae) is commonly used as Java Tea. It is a medicinal plant, native in South East Asia (Malaysia, Indonesia, and Thailand) and some part of Tropical Australia [1]. O. stamineus is popularly
consumed as herbal tea and is one of the popular traditional folk medicine extensively used in Southeast Asia for the treatment of wide range of diseases including diabetes, kidney and urinary disorders, high blood pressure and bone or muscular pain [2–5]. In the present study, we studied possible anticancer properties of *O. stamineus* decoction on hepatocellular carcinoma in animal model. Moreover, apart from different cancer markers which have been assessed, due to importance of glucocorticoids in cancer, effect of *O. stamineus* decoction on serum glucocorticoid as well as liver cell glucocorticoid receptors were investigated.

**Methods**

*Animals and experimental protocols*

The present study was designed as a preclinical study. The protocol of the rat hepatocarcinogenesis in this study was based on Solt and Farber method [6]. University Putra Malaysia (UPM) Animal Research Ethics Committee has approved this study under approval No: UPM/FPSK/PADS/BR-UUH/00448. Forty male Sprague Dawley rats, 8±1 weeks old, with average weight 246.3±6.2 g, were obtained from UPM Veterinary Faculty and housed in individual plastic bottom cages and maintained in a room at 22 ºC temperature, humidity 60±5% relative with a 12h light/dark cycle. All rats had free access to the standard rat food pellet based on AIN-76A [7], and tap water during the study. Hepatocarcinoma were induced in 30 of the rats by single intraperitoneal injection of 200mg/kg body weight diethyl nitrosamine (DEN) dissolved in corn oil and then were kept for 2 weeks on cancer promoter food, which was mixed with 2-acrylamidofluorene (0.02% AAF) as a promoter of hepatocarcinogenesis without partial hepatectomy to promote hepatocarcinogenesis. The rats were then left for 2 weeks. A group of 10 rats served as normal group with no DEN injection or hepatocarcinogenesis promoter diet. After cancer initiation period, the left over rats were weighed again and divided randomly to two groups with no significant differences in their weight. Both control and *O. stamineus* group were allowed free access to AIN76 and water *ad libitum* for 28 weeks, but rats in *O. stamineus* group were force feed by 0.7ml/100g Body weight/day of *O. stamineus* decoction.

**Plant material and Preparation of the *O. stamineus* decoction**

*O. stamineus* was prepared from University Agriculture Park of UPM. *O. stamineus* leaves were dried under shadow, weighed and washed 3 times with tap water and then put into a 1 liter beaker. For each 100g of dried herb, 4000 ml of distilled water was added. Then the mixture was heated up to 70°C to decrease the water content to 1000 ml through evaporation. After these steps, the residues were filtered. The liquids were cooled and kept in the fridge at 4°C in clean bottles until used. This process has been done weekly until end of the study.

**Chemicals and Biochemical analyses**

Alpha-Fetoprotein tumor marker (AFP), tumor necrosis factor alpha (TNF-α), homocysteine (HCY), corticosteroid binding globulin (CBG), alpha 2 macroglobulin (α2-MG) were analyzed using standard commercial ELISA kit (Cusabio Biotech, China). Gamma glutamyl transpeptidase (GGT) were tested by using Colorimetric Assay Kit (BioVision, USA). Alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), alkaline phosphatase (ALP), and total antioxidant status (TAS) were analyzed by Chemical pathology lab at FMHS, UPM using Roche Cobas® C-311 analyzer.

**Histopathological examinations**

Half of the liver tissue of each samples were fixed in 10% formalin and then the paraffin blocks were prepared. The sections from blocks were stained with hematoxylin-eosin. The histopathological evaluations were performed blindly by an expert pathologist using a scoring system. The lesion scoring was obtained done weekly until end of the study.

**Results**

Alpha-Fetoprotein tumor marker (AFP), tumor necrosis factor alpha (TNF-α), homocysteine (HCY), corticosteroid binding globulin (CBG), alpha 2 macroglobulin (α2-MG) were analyzed using standard commercial ELISA kit (Cusabio Biotech, China). Gamma glutamyl transpeptidase (GGT) were tested by using Colorimetric Assay Kit (BioVision, USA). Alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), alkaline phosphatase (ALP), and total antioxidant status (TAS) were analyzed by Chemical pathology lab at FMHS, UPM using Roche Cobas® C-311 analyzer.
Table 1. Effect of *O. stamineus* on serum biochemical markers as compared to normal and control groups

<table>
<thead>
<tr>
<th>Marker</th>
<th>Normal (n=10)</th>
<th>Control (n=8)</th>
<th><em>O. stamineus</em> (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (IU/L)</td>
<td>38.86±1.72 a</td>
<td>77.92±3.74 b</td>
<td>59.00±6.70 c</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25.89±1.66 a</td>
<td>67.33±4.92 b</td>
<td>41.07±3.80 c</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>56.73±2.53 a</td>
<td>156.49±10.66 b</td>
<td>106.19±16.62 c</td>
</tr>
<tr>
<td>AST/ALT Ratio</td>
<td>2.25±0.08</td>
<td>2.48±0.25</td>
<td>2.60±0.34</td>
</tr>
<tr>
<td>CBG (μg/ml)</td>
<td>10.76±0.26 a</td>
<td>11.52±0.35 b</td>
<td>12.53±0.45 c</td>
</tr>
<tr>
<td>HCY (nmol/ml)</td>
<td>0.57±0.03 a</td>
<td>1.42±0.14 b</td>
<td>0.94±0.04 c</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>24.08±1.00 a</td>
<td>49.17±1.12 b</td>
<td>43.97±1.63 c</td>
</tr>
<tr>
<td>α2MG (ng/ml)</td>
<td>0.71±0.04 a</td>
<td>1.43±0.09 b</td>
<td>1.23±0.16 c</td>
</tr>
<tr>
<td>AFP (pg/ml)</td>
<td>47.6±1.05 a</td>
<td>102.05±2.86 b</td>
<td>83.99±4.10 c</td>
</tr>
<tr>
<td>TAS (mmol/l)</td>
<td>9.86±0.35 a</td>
<td>1.09±0.16 b*</td>
<td>8.98±0.18 c</td>
</tr>
<tr>
<td>GGT (mU/ml)</td>
<td>0.68±0.01 a</td>
<td>1.14±0.04 b</td>
<td>0.84±0.015 c</td>
</tr>
</tbody>
</table>

Values in the same row with the different superscripts are significantly different at *p*<0.05 based on one way ANOVA, Duncan’s post hoc test.

* Values are significantly lower than other groups at *p*<0.01 based on one way ANOVA, Duncan’s post hoc test.

Data were expressed as Means±SEM. Statistical differences between Normal, treated and control groups were determined using one way repeated measures analysis of variance (ANOVA) followed by Duncan’s multiple range as post hoc test. Differences between groups were considered significantly different when the P value was less than 0.05.

**Results**

During the intervention study some of the rats in both groups died. *O. stamineus* treated group showed non-significantly lower mortality rate as compared to the control group (25% vs 33.3%, *p*<0.05). After 28 weeks of treatment, based on the present findings, rats in the control group showed higher but no significant weight gain as compared to *O. stamineus* group 491.2±17.9 g vs 461.1±15.0 g (*p*>0.05). Although the control group showed significantly higher liver weight (12.28±0.31g) as compared to both normal (10.50±0.38g) and *O. stamineus* group (10.94±0.63g) (*p*<0.05), no significant liver weight ratio was observed 2.47±0.06 vs 2.60±0.17 and 2.28±0.10 respectively (*p*>0.05).

The effect of different treatments on serum biochemical biomarkers has been demonstrated in Table 1. Both normal and *O. stamineus* groups showed significantly lower serum ALP (*p*<0.05) as compared to the control group. Similar outcomes were also found for AST, ALT, HCY, and α2MG (*p*<0.05). In spite of lower level of

**Figure 1.** Percentage of changes of different serum biochemical markers in *O. stamineus* group as compared to the control
AST/ALT ratio in normal group, no significant difference was found between groups (p>0.05). Promising considerably higher level of CBG was found in O. stamineus group and the lowest value was found in normal group (p<0.05). O. stamineus showed significantly higher level of TNF-α, α2MG, AFP, and GGT as compared to normal (p<0.05) but significantly lower than control group (p<0.05). O. stamineus group showed significantly higher TAS as compared to the normal group (p<0.05), and both were considerably higher than control group (p<0.01).

As Figure 1 indicates, after 28 weeks of treatment with O. stamineus decoction most of liver function and cancer markers have improved and fallen significantly as compared to the control (p<0.05). ALP was reduced by 24.28±1.79 %, ALT, AST, and HCY decreased by 39±0.77%, 32.14±1.56%, and 33.8±0.29% respectively. AST/ALT ratio was increased nonsignificantly 4.84±1.36, (p>0.05). TNF-α, α2MG, AFP and GGT were also dropped (10.58±1.46%, 13.99±1.78%, 17.7±1.43%, 26.67±0.38% respectively). Unlike other markers, CBG has elevated significantly (8.77±1.29%, p<0.05). Moreover, O. stamineus decoction was able to upsurge TAS drastically by 723.85±1.13%.

**Histopathological findings**

Lesion score of different group is shown in Figure 2. Accordingly, rats in control groups showed significantly higher lesion score in both portal and lobular region as well as fibrosis stage.

![Figure 2](image_url)

**Figure 2. Mean lesion score of rats liver tissue in different groups**

abc Values in each area/stage with the different superscripts are significantly different at p<0.05, based on one way ANOVA, Duncan's post hoc test.

![Figure 3](image_url)

**Figure 3. Light micrograph of liver cells in different groups.**

compared to *O. stamineus* and normal groups (p < 0.05). Obviously, normal group had the lowest lesion score (p < 0.05) due to rats health status. *O. stamineus* group showed significantly lower lesion score in all sites as compared to the control group (p < 0.05) (Figure 3).

**Discussion**

In this study, the control group had the highest mortality rate, which was expected. Although gross histology of *O. stamineus* group showed no hepatic nodules in this group unlike the control ones, quite similar mortality rate was a disappointing outcome. Although significantly higher weight in control group was found which was in contrast with previous studies [10], the relationship between HCC and body weight is still questionable and there are many different opinions on this topic. Usually weight loss would be seen in critical level of liver cancer [11]. There might be many possible underlying variables, which can affect weight loss or gain in liver cancer. Disease acquaintance and severity of the disease are two main factors which mainly dominate weight changes. On the other hand mortality rate in groups might affected the real result of body weight in the present study. The result of liver weight of *O. stamineus* was also in contrast with previous studies in close field [10]. Differences in study design as well as duration of the studies might be the reason of these differences. It has been shown that ALP among liver function tests, in addition to other tumor characters, were independent factors for disease-free survival and overall survival. Recent studies have suggested that preoperative ALP levels could be utilized to monitor and predict recurrence in high risk HCC patients [12]. Both Normal and *O. stamineus* groups showed significantly lower serum ALP (p < 0.05) which was similar to previous studies (10,13). Significant elevation of serum AST and ALT activities were seen in a variety of liver conditions, cirrhosis, non-alcoholic steatohepatitis (NASH), drug toxicity, liver tissue degeneration and necrosis [14]. AST elevations frequently predominate in patients with cirrhosis and even in liver diseases that classically have an increased ALT [15]. Both normal and *O. stamineus* groups showed significantly lower serum AST and ALT (p<0.05). Advantageous effect of *O. stamineus* on liver enzymes including AST and ALT has been reported previously [10,13,16] and the present study supported the previous claims as well. Concerning AST/ALT ratio which has been reported a more clinical utility than assessing individual elevated levels [17], non-significant lower ratio was found among *O. stamineus* group. Absence of significant result might be due to higher mean standard error among control group.

The role of TNF-α in liver cancer has dual capabilities. TNF-α is a pleiotropic cytokine that can induce both cell death and cell proliferation [18]. It is related to all steps engaged in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [19]. Deregulated TNF-α expression within the tumor microenvironment seems to favor malignant cell tissue invasion, migration and finally metastatic formation. On the other hand, TNF-α clearly possesses antitumor properties not only in

![Figure 4](image-url) Fluorescent in situ hybridization micrograph of lobular region of protein expression of glucocorticoid receptors in the cytoplasm. A: Normal, B: Control. C: *O. stamineus* treated group. Frozen section, ×600 Magnification. More green color express more GR.
preclinical models but also in the clinical setting [20]. Overall, based on the present study, O. stamineus can decrease serum TNF-α either due to direct effect of its two main compounds eupatiorin and sinensetin, which inhibit TNF-α production [21] or indirect mechanism by suppressing HCC.

Animal studies showed that αMG is an important novel cytochemical marker to identify hepatocellular preneoplastic and neoplastic lesions, particularly amphophilic cell foci, undetectable by established cytochemical markers and tightly linked to rat hepatocarcinogenesis [22]. Furthermore, a number of authors have reported up-regulation of serum αMG in association with HCC in humans, being significantly raised as compared to liver cirrhosis and amoebic liver abscess [23]. In the present study, O. stamineus treated group showed significantly lower level of αMG. As cancer cells produce and secrete large amounts of αMG, which seems to be linked with their tumorigenicity [24], therefore O. stamineus might decrease αMG secretion in cancer suppression mechanism.

Concerning HCY outcome, the present results are supported by a few studies which have found high levels of HCY in different types of cancer [25] and liver disorders [26]. Both epidemiological and experimental studies found a link between hyperhomocysteinemia and a wide range of impaired liver function like cirrhosis and chronic alcohol consumption [27]. To our knowledge the present study was the first attempt to investigate the effect of treated herb on HCY level, therefore there was no pros and cons evidence to the present results.

AFP is one of the old, yet the most widely used blood marker test for liver cancer. High level of AFP among control group of the present study was similar to previous ones. Many of the studies in last four decades have shown that AFP was elevated in hepatocarcinogenesis, embryonic carcinomas [28,29]. Beneficial effect of O. stamineus in the present study might be due to antioxidant activity and flavonoids in O. stamineus [30–32].

It is well established that the elevated serum GGT activity could be found in diseases of the liver, biliary system, pancreas, and different types of cancers including HCC [33]. In the present study, significantly lower level of GGT was found in O. stamineus group as compared to control group (p<0.05). It has been shown that GGT is associated with antioxidants and oxidative stress [34]. Extremely high level of TAS in treated group could be one of the master keys in revealing possible cancer suppressor capabilities of O. stamineus. It has been shown that excessive reactive oxygen species (ROS) cause oxidative damages to biomolecules and lead to cellular alterations and ultimately tumorigenesis and neoplastic transformation [35]. Therefore, high level of TAS could not only act as excess ROS protector, but might also indirectly affect other cancer risk factors which have been tested.

Glucocorticoids (GCs) are frequently used to support patients suffering from a various type of cancers. Their key therapeutic role is based on GC receptor (GR)-mediated mechanisms that activate cell death however this differs depending on type of cancer [36]. GC might directly activate the apoptotic machinery by regulating components of either the ‘extrinsic’ or ‘intrinsic’ pathways or both. Studies using the caspase 8 inhibitor cytokine response modifier A (CrmA) in transgenic mice [37] and human cell lines [38] suggested that GC-induced apoptosis may not critically depend on the extrinsic pathway. GC might induce apoptosis indirectly by gene (de)regulations that entail distress and cellular damage. This category might include the regulation of genes affecting metabolic pathways [39,40], general transcription and/or translation [41], production of/or response to oxygen radicals [42,43]. Moreover, glucocorticoids prevent prostaglandin synthesis at the level of phospholipase A2 as well as at the level of cyclooxygenase/PGE isomerase (COX-1 and COX-2) [44]. The latter effect is similar to non-steroidal anti-inflammatory drugs (NSAIDs), which potentiate the anti-inflammatory effect [45,46]. COX-2-dependent activity is an essential element for cellular and molecular mechanisms of cancer cell motility and invasion. COX-2 activity also modulates the expression of matrix metalloproteinase (MMP), which may be a part of the molecular mechanism by which COX-2 promotes cell invasion and migration [46]. Many studies on different type of cancer have shown that cyclooxygenase suppression would decrease cancer cells [45]. Therefore, cyclooxygenase suppression by glucocorticoids might decrease risk of cancer or control its metastasis. Glucocorticoids inhibit hepatocellular proliferation and modulate the expression of oncogenes and tumor suppressor genes via mechanisms involving the glucocorticoid receptor. Glucocorticoids also
produce a receptor-mediated inhibitory effect on both basal and hormone-stimulated expression of molecules important for shutting off cytokine action as well as different caspase pathways [47]. Based on the present study, *O. stamineus* has glucocorticoids stimulation activity, which might have positive effect on cancer prevention or treatment. The results of confocal microscopy of fluorescent *in situ* hybridization of liver cells helped us to have a better answer for our findings in both light microscopy as well as biochemical results. As FISH results shown in Figure 4, high level of glucocorticoid receptor activity was illustrated in *O. stamineus* treated group. Therefore, higher activity of GC receptors and higher level of serum CBG which have been found in the present study could also explain the possible anti-cancer or cancer suppressor competences of *O. stamineus*. Overall, as the present study is the first study in its field further studies would help us to have better view on mechanism of action of these herbs on glucocorticoids stimulation.

Lesion score evaluation of rat livers also showed *O. stamineus* decoction successfully reduced the score of inflammation or necrosis at the portal and lobular as compared to control group (p< 0.05) which was similar to previous studies [10,13].

**Conclusion**

These beneficial effects of *O. stamineus* could be explained by significantly high level of flavonoid compounds and antioxidant capability of the *O. stamineus*. In order to find out the active compound(s) that play major role(s) in producing these effects, further studies are necessary.

**Acknowledgements**

The authors would like to thank Mrs. Safarina Ismuuddin, Mrs. Zamzarin Bajari, Mr. Norazmie Ramly, Mr. Syed Hasbullah Syed Kamaruddin, Mr. Mohd Khairil, and Mr. Ramli Suhaimi for their technical assistance. This study was funded by Universiti Putra Malaysia under RUGS-9367600 research grant.

**Conflicts of interest:** The authors of this article declare that they have no conflict of interest in the subject matter or materials discussed in this manuscript.

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