IN VITRO EVALUATION OF ANTIDIABETIC AND CYTOTOXICITY POTENTIALS OF THE RHIZOME EXTRACT OF DRYNARIA QUERCIFOLIA (L.) J. SMITH

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INTRODUCTION

Diabetes mellitus (DM) is one of the metabolic and endocrinological disorders [1-3] which results from an irregularity in insulin secretions and insulin actions or both. Absence or reduced insulin secretion leading to persistence of high glucose level and glucose intolerance [4]. Diabetes is generally accepted as a major challenging health problem all over the world and especially in the developing countries. 3.5 million deaths occur globally by diabetes [5]. Insulin therapy, oral hypoglycemic agents, restricted diet, exercises either singly or in combination constitute a major regimen of therapy available for the present day diabetes. Although in modern medicine, there is no satisfactory effective therapy available to cure Diabetes Mellitus. In a many numbers of cases, treatment with traditional medicine in the form of plant extracts has been reported to give remarkable good results [6]. Plant and plant products played an important role in the treatment of DM and had been used throughout the world since ancient times. Folk and traditional medicine healing system has been used globally as an antioxidant, antihyperglycemic, and antidiabetic properties from plants [7]. Moreover, since the herbal products are coming from biological origin, they can subside the limitations arising from the use of conventional hypoglycemic.

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, and it may cause death worldwide. According to a recent estimate, about 782,000 people were diagnosed with liver cancer, out of which 746,000 people died [8]. A total of 30,640 new liver and intra hepatic bile duct cancer were estimated in 2013 in addition to the 21,670 deaths [9]. The efficacies of chemotherapy, radiotherapy, hormonal therapy, or surgery, which are mainly used for the treatment of cancer, are well-known for side effects [10]. Hence, the identification of novel natural products that possess better effectiveness against cancer, but less harmful effects have become desirable [11], and therefore, natural products are continuously being explored worldwide. Keeping the above states in mind, present study has been designed to assess the antidiabetic and cytotoxicity potentials of the rhizome extract of medicinal fern, Drynaria quercifolia (L.) Smith by in vitro assays.

Drynaria, commonly known as basket ferns, is a genus of ferns in the family of Polypodiaceae. It contains around 16 species. They are found in wet tropical environments, usually in rainforests [12]. D. quercifolia contains various phytochemicals such as phenols, tannins, alkaloids, proteins, xanthoproteins, carboxylic acid, coumarins, saponins, catechin, flavonoids, steroids, and triterpenes [13]. Phytochemicals such as friedelin, epifriedelin, beta-amyrin, beta-sitosterol, beta-sitosterol 3-beta-D-glucopyranoside, 3, 4 di hydroxyl benzoic acid, acetyl lupeol, aglycone naringenin, and flavones glycoside naringin were identified in dried rhizomes of D. quercifolia. Many pharmacological activities have been reported such as antimicrobial [14], antidermatophytic [15], antioxidant [16-18], hepatoprotective [19], anti-inflammatory and antipyretic [20], analgesic [21], and anti-ulcer [22] activities.

METHODS

Collection of plant material

The rhizomes of D. quercifolia (L.) J. Smith was collected from Kollimalai, Namakkal district, Tamil Nadu, India. The collected rhizomes were carefully bagged in polythene bags. These plant samples were authenticated by Dr. S. John Britto, Director, RAPINAT Herbarium, St. Joseph’s College, Tiruchirappalli, and a voucher specimen was deposited in the Department of Biochemistry, S. T. E. T. Women’s College, Mannargudi (Voucher No...001).

Extract preparation

10 g of the powdered sample was extracted with 85% methanol using Soxhlet extraction method. The filtrate was condensed using rotary vacuum evaporator at 45°C and the extract was stored at 4°C until used.

ABSTRACT

Objective: In the present study, an attempt has been made to evaluate the in vitro antidiabetic and cytotoxic potentials of the rhizome extract of Drynaria quercifolia (L.) J. Smith.

Methods: In vitro antidiabetic activity was determined by two different assays such as alpha-amylase inhibition assay and glucose uptake assay. The plant extract with three different concentrations was used for this assay. L6 rat myogenic cells were selected and subjected to glucose uptake assay. The cytotoxic activity of the different concentrations of the plant extract on HepG2 cell line was also investigated in vitro through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Results: The findings of the study provide evidence that the rhizome extract of D. quercifolia possesses significant anti-diabetic activity. In MTT assay, the significant cytotoxic effect of plant extract was observed by measuring the percentage of cell viability on the HepG2 cell line.

Conclusion: The findings indicated that rhizome extracts of D. quercifolia have potential as a medicinal drug against diabetes mellitus (DM) and liver cancer. Further, studies with in vivo and clinical trials need to be conducted to establish rhizome extract as a safe agent for DM and liver cancer therapy.

Keywords: Anticancer, Antidiabetic, Cytotoxicity, Drynaria quercifolia, HepG2, L6 rat myogenic cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, Rhizome extract.
Antidiabetic assay

L6 rat myogenic cells

L6 rat myogenic cells were cultured in hypotonic buffer (20 mM Tris-HCl, pH 8.1, 1 mM ethylenediaminetetraacetic acid, 0.2 mM EGTA, 50 mM NaF, 0.7 μg/ml peptatin, 10 mM sodium orthovanadate, and 50 mM benzamidine, 0.5 μg/ml leupeptin, 4 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride). The cell suspension was centrifuged at 20,000×g for 15 min and homogenate obtained. The supernatant was collected as the soluble fraction for further experiments.

Preparation of test solutions

The methanol extract of D. quercifolia was separately dissolved in distilled dimethyl sulfoxide (DMSO) and the volume was made up with medium supplemented with 2% inactivated fetal bovine serum (FBS) to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. From this stock solution, four different lower dilutions (5.0, 10.0, 20.0, and 40.0 μg/ml) were prepared and subjected to glucose uptake assay.

Alpha-amylase inhibition assay

The plant extract with three different concentrations (25, 50, and 100 μg/ml) were used for this assay. Plant extract a total of 500 μl and 0.02 M sodium phosphate buffer of 500 μl of pH 6.9 with 0.006 M sodium chloride containing α-amylase solution (0.5 mg/ml) were incubated for 10 min at 25°C after pre-incubation, 500 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5 s intervals. This reaction mixture was then incubated for 10 min at 25°C. 1 ml of DNA reagent was added to stop the reaction. After that, all the test tubes were incubated in a boiling water bath for 5 min and cooled at room temperature. Finally, this mixture was again diluted with 10 ml distilled water the absorbance was measured at 540 nm [23].

Glucose uptake assay

L6 rat myogenic cells were selected and seeded into 96-well plate with six wells left as blank wells and let growing to confluence; then, cells were fully differentiated in DMEM with 2% FBS for 5 days. Before tests, the medium was replaced by RPMI-1640 (2 g/L glucose) supplemented with 0.2% BSA. The medium was removed after 2 h, and the same medium containing plant extract (5.0, 10.0, and 40.0 μg/ml), metformin (0.01 mM) the standard, and DMSO was added to all wells in the absence or presence of insulin (1 μmol/L). Blank was also performed where it is reduced to an insoluble, colored (dark purple) formazan product.

RESULTS AND DISCUSSION

DM is a leading metabolic disorder in worldwide and India is the capital for diabetes. Diabetes is a condition which is caused by defective synthesis of or defective function of insulin, the body does not produce enough insulin or properly respond to insulin. Insulin is a hormone which is produced in β cells of the pancreas and it stimulates the body cells to absorb glucose from the blood. DM is one of the most common endocrine and metabolic disorders which cause various microvascular complications such as retinopathy, nephropathy, and macrovascular complications such as heart attack, stroke, and peripheral vascular diseases. Nearly 2.9% of the world’s population affected by diabetes and it is expected to increase up to 5.4% in the year 2025 [27]. Various kinds of antidiabetic therapies such as sulfonylureas, biguanides, and glinides are available, but it causes various adverse effects. Hence, the researchers are investigating and try to find more effective and safer hypoglycemic agent without causing any side effects [28].

Nowadays, people are turning toward herbal remedies due to fewer side effects with comparing to the oral hypoglycemic agents for the treatment of DM [29]. It is estimated that more than a thousand plant species are being used as folk medicine for diabetes [30]. Biological actions of the plant products used as alternative medicines to treat diabetes are in relation to their phytoconstituents. Phytoconstituents such as flavonoids, phenolic compounds, coumarins, terpenoids, and other constituents help to reduce blood glucose levels [31].
In vitro assay, to evaluate the antidiabetic activity of rhizome of *D. quercifolia*. Obtained results were recorded and discussed here.

One-way of controlling Type 2 DM (T2DM) is to limit intestinal carbohydrate digestion. Although the gastrointestinal tract does not play a major role in the pathogenesis of T2DM, modification of its physiological activities can be used to decrease post-prandial hyperglycemia through inhibition of the carbohydrate hydrolyzing enzymes, α-amylase, and α-glucosidase [34,35]. In our study, different concentrations (25, 50, and 100 µg/ml) of methanol extract of selected plant parts were subjected to alpha-amylase inhibition assay. Metformin was used as standard. Inhibition percentage of plant extract was found to be 40.1±2.6, 65.7±4.1, and 79.8±3.5 at concentration of 25, 50, and 100 mg/ml, respectively. For standard drug, it was 85.0±7.6 (Table 1).

In general, in vitro antidiabetic assays help to assess the effects of compounds on the inhibition of the two key enzymes involved in carbohydrate metabolism, namely, α-amylase and α-glucosidase, which has been described by several authors [36,37]. Inhibition of these enzymes results in a delay in the degradation of starch and oligosaccharides, which decreases the absorption of glucose and consequently inhibits post-prandial hyperglycemia [38]. In humans and other mammals, the maintenance of plasma glucose concentration for the long term is one of the most important and closely regulated processes observed under a variety of dietary conditions [39].

In the present study, there was a dose-dependent, alpha-amylase inhibition was observed in plant extract-treated cells. That the concentration was increased, inhibition activity of alpha-amylase was also significantly (p<0.001) increased. By comparing with standard, plant extract produced moderate inhibition. Metformin is one popular drug used in the treatment of DM. It causes a variety of side effects such as gastrointestinal upset, lactic acidosis, and loss of weight [40]. On the other hand, medicinal plants have much less side effects and are thus being preferred over synthetic drugs in our modern health-care system.

Adipose tissue, skeletal muscle, and the liver are the three major target tissues of insulin. T2DM is characterized by insulin resistance and an insulin secretion deficiency [41,42]. The latter result in a reduction of glucose uptake to adipose tissue and skeletal muscle, as well as it increases gluconeogenesis in the liver. In *in vitro* assay, cell lines, such as L6 (rat myoblasts), C2C12 (mouse myoblast), 3T3-L1 (mouse fibroblast), HepG2 (human hepatocarcinoma), and Chang liver cells (human liver) are commonly used to assess glucose uptake. Glucose uptake assays assess the ability of the experimental compounds to enhance glucose uptake in the major target tissues of insulin, represented by the cell lines. In the present study, insulin sensitization effect of selected plant extract was examined in L6 rat skeletal muscle cells through glucose uptake action to confirm the possible antidiabetic mechanism of plant extract.

In this assay, incubation of *D. quercifolia* extract (5.0, 10.0, 20.0, and 40.0 µg/ml), and metformin (0.01 mM) in muscle cells in the presence of insulin (1.13 µmol/L) showed significant (p<0.001) glucose uptake action when compared to vehicle control. In the presence of insulin, glucose uptake was 6.13±0.28, 6.23±0.19, 6.39±0.54, and 6.73±0.41 at a concentration of 5.0, 10.0, 20.0, and 40.0 µg/ml of plant extract, respectively. Similarly, in the absence of insulin, it was found to be 5.02±0.32, 4.98±0.27, 4.06±0.16, and 3.16±0.21. Standard metformin has the glucose consumption of 7.07±0.30 and 4.81±0.31 in the presence and absence of insulin, respectively (Table 2).

Two main important features observed in type 2 diabetes and they are peripheral insulin resistance and impaired insulin secretion from pancreatic β-cells. Liver, skeletal muscle, and adipose tissue are the peripheral tissues in which resistance of insulin generally occurred. Hyperglycemia and dyslipidemia are interlinked disorders in type 2 diabetic patients commonly cause the occurrence of cardiovascular diseases which mainly due to insulin resistance. Hyperlipidemia is one of such common complication of diabetes, which is characterized by increase in serum total cholesterol, triglycerides, low-density lipoprotein (LDL), and very LDL [43]. Drugs such as metformin diminishes insulin resistance as well as effectively control hyperglycemia and ameliorate lipid metabolism in type 2 diabetes, hence it prevents diabetes-mediated cardiovascular complications [44]. This class of drugs has adverse effects such as lactic acidosis, gastrointestinal disturbance, liver toxicity, and cardiovascular risk [41]. Thus, for long-term treatment in type 2 diabetes, drugs which improve insulin sensitivity without adverse effects were reported to be useful.

In the present study, we examined glucose uptake action of rhizome extract of *D. quercifolia* in L6 rat skeletal muscle cells; the results revealed that standard drug metformin significantly (p<0.001) enhanced glucose uptake activity which was higher than plant extract in the presence and absence of insulin. Plant extract did not exhibit higher glucose uptake action like standard drug metformin in the absence and presence of insulin. However, results confirmed that plant extract enhanced glucose uptake activity in the presence of insulin than the absence of insulin when compared to vehicle control and also plant samples produced dose-dependent glucose uptake action. The plant drug effectively prevents hyperglycemia and thereby it controls lipid metabolism.

This data have given clear evidence that the selected plant part possibly acts to improve the glucose uptake in the skeletal muscle in the presence of insulin, and hence it has potential to reverse insulin resistance in type 2 diabetes. It also suggests that the plant extract has insulin-nimetic properties and could be a potential antidiabetic drug which could alleviate hyperglycemia by enhancing glucose uptake.

### Table 1: Alpha-amylase inhibition of *Drynaria quercifolia* rhizome

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µg/ml)</th>
<th>% of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0±0</td>
</tr>
<tr>
<td>Plant extract</td>
<td>25</td>
<td>40.1±2.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>65.7±4.11*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>79.8±3.5*</td>
</tr>
<tr>
<td>Metformin</td>
<td>0.1</td>
<td>85.0±7.6*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of mean (n=3); values not sharing common superscript were statistically significant with from each other (p<0.001)

### Table 2: Glucose uptake assay of *Drynaria quercifolia* rhizome on L6 rat muscle cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µg/ml)</th>
<th>Absence of insulin</th>
<th>Presence of insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.18±0.06</td>
<td>5.67±0.50**</td>
</tr>
<tr>
<td>Plant extract</td>
<td>5.0</td>
<td>5.02±0.32</td>
<td>6.13±0.28*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.98±0.27</td>
<td>6.23±0.19</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.06±0.16</td>
<td>6.39±0.54*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.14±0.21</td>
<td>6.73±0.41*</td>
</tr>
<tr>
<td>Metformin</td>
<td>0.1 µM</td>
<td>4.81±0.31*</td>
<td>7.07±0.30</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of mean (n=3); values not sharing common superscript were statistically significant with from each other (p<0.001)

**MTT assay**

Primary liver cancer (PLC) has two major forms which are of HCC and intrahepatic cholangiocarcinoma, accounting for approximately 90% and 5%, respectively [45]. HCC is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. HCC is now the third leading cause of cancer deaths worldwide, with over 500,000 people affected. The induction of HCC is preceded by the occurrence of hepatocellular damage through reactive
oxygen species (ROS) and the generation of chronic inflammation related to hepatic carcinogenesis [46].

Over the years, different approaches have been employed and are still in use, individually or in combination, in the treatment of cancer such as chemotherapy, radiotherapy, surgery, and immunotherapy. Chemotherapeutic agents are cytotoxic to other than cancer cells and proliferate them into cancer cells, including those localized in the gastrointestinal tract, hair, and bone marrow, thus eliciting the gastrointestinal side effects such as nausea and vomiting, abpecia, and myeloid suppression. Anticancer cells have limitations in their efficacy due to insolubility and instability, the low rate at which the tissue absorbs them, and tumor drug resistance [47]. In addition, antitumor drugs have also been associated with the development of secondary malignancy. All the drawbacks presently associated with available chemotherapeutic agents are the impetus for the search for newer, more efficacious, and better-tolerated drugs.

Plants have a long history of use in the treatment of cancer [48,49] and the interest in nature as a source of potential chemotherapeutic agents continues. Recently, researchers put steps forward at herbal plants for the discovery of new antiproliferative agents with safety and efficacy. Hence, in the present study, we evaluate in vitro cytotoxic potential of medicinal plant D. quercifolia on HepG2 cell lines by MTT assay.

Different concentrations of 25, 50, 100, 200, and 400 μg/ml of rhizome extract of D. quercifolia were subjected to evaluate anticancer activity against HepG2 cell lines by MTT assay. Standard doxorubicin was also evaluated to compare the efficacy of anticancer activity of selected plant extract. Percentage of viability was found to be 85.8±5.4 at 25 μg/ml followed by 76.7±6.9 at 50 μg/ml, 68.1±5.3 at 100 μg/ml, 58.3±4.9 at 200 μg/ml, and 35.6±2.8 at 400 μg/ml (p<0.001). For standard drug, it was 24.3±1.5 μg/ml. The percentage viability was increased with decreasing concentration of test compounds (Table 3). Thus, cancer cell growth was inhibited maximum at high concentration of plant extract, but it was less than that of standard.

The MTT assay is a sensitive, quantitative, and reliable colorimetric assay that measures viability, proliferation, and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate MTT into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation, which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells were dead following toxic damage, cannot transform MTT. This formation production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity. In the present study, percentage of viability was increased when the concentration of plant extract was decreased. That is cell viability was increased at the concentration of 25 μg/ml then it was significantly decreased with increased concentration of extract.

Several reports described that the anticancer activity of the medicinal plants which may be due to the presence of antioxidants (namely, vitamins, carotene, enzymes, minerals, polysaccharides, polyphenols, flavonoids, lignins, and xanthones). In addition, it was reported that these plants may promote host resistance against infection by re-stabilizing body equilibrium and conditioning the body tissues [50]. Our result suggests that D. quercifolia may be a potential chemotherapeutic or a chemopreventive agent based on its ability to induce apoptosis in cancer cells with relatively low toxicity to normal cells.

CONCLUSION

From the present work, it was concluded that the selected medicinal fern D. quercifolia possesses remarkable pharmacological activities toward DM and HCC, which will claim their folk-lore uses as antidiabetic and cytotoxic agents. Further, studies on in vivo action and isolation of the principle bioactive constituent(s) are also needed.

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AUTHORS’ CONTRIBUTIONS

All the authors contributed equally to the paper.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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14. Nejad BS, Deokule SS. Anti-dermatophytic activity of Drynaria quercifolia (L.) J. Smith, Sundankottai, TN, India, for their support to carry.
15. Nejad BS, Deokule SS. Anti-dermatophytic activity of Drynaria quercifolia (L.) J. Smith, Sundankottai, TN, India, for their support to carry.

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**Table 3: MTT assay of Drynaria quercifolia rhizome against Hep G2 cell lines**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Conc. (μg/ml)</th>
<th>Absorbance at 570 nm</th>
<th>% cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HepG2 cells untreated</td>
<td>-</td>
<td>0.28</td>
<td>100.0±7.5**</td>
</tr>
<tr>
<td>2</td>
<td>Plant extract treated</td>
<td>25</td>
<td>0.24</td>
<td>85.9±5.4*</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>50</td>
<td>0.22</td>
<td>76.7±6.9*</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>100</td>
<td>0.19</td>
<td>68.1±5.3*</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>200</td>
<td>0.17</td>
<td>58.3±4.9*</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>400</td>
<td>1.10</td>
<td>35.6±2.8*</td>
</tr>
<tr>
<td>7</td>
<td>Doxorubicin treated</td>
<td>100</td>
<td>0.07</td>
<td>24.3±105*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±standard error of mean (n=3). Values not sharing common superscript were statistically different from each other (p<0.001).

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.


