Nephrotoxic potential of *Carex baccans* (Family: Cyperaceae): A light and electron microscopic studies

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Abstract  
*Carex baccans* is used widely as anthelmintic medicinal plant in different part of Northeast India. The present study was carried out to evaluate the extent of kidney damage in albino rats, if any due to consumption of high doses of extract of *C. baccans*. Rats were administered orally with the plant extract at doses of 40, 100, 200 and 400 mg kg$^{-1}$ body weight at a single dose per day, for a period of 2 weeks. At the end of experiment, the animals were sacrificed and the kidneys were processed for light and transmission electron microscopic observations. Exposure of plant extract resulted in distinct histological and ultrastructural changes in podocytes, mesangial cells, juxtaglomerular cells and glomerular basement membrane. While DAPI stained nucleus showed condensation of nucleus; TUNEL staining confirmed the apoptotic nature of cell death in extract exposed rats. These results suggest that exposure of rat to crude extracts of *C. baccans* may induces apoptosis in kidney, resulting in altered structural and functional integrity of kidney. Further studies involving active principle(s) of the plant responsible for kidney damage would help in understanding the public implication of its consumption in the region.

Citation:  

1. Introduction

1.1 Traditional value  
Traditional way of healing involving phytoproducts is considered to play a vital role in rural healthcare system, particularly in developing countries, because of its low cost, easy availability and a belief that plant products are free from undesired effect on humans. Demand for plant based treatment is gradually increasing not only in developing countries, but also in developed nations (Li et al., 2009). According to World Health Organization (WHO) about 80% of global population meets their key healthcare needs through traditional medicines (Fiorentino et al., 2008). However, recent investigations showed that several medicinal plants exert toxic effect on animals and men in the form of allergic reactions to cardiovascular, hepatic, renal, neurological and dermatological systems (Vahid et al., 2006). In this respect it is observed that a lot of effective antiparasitic medicinal plants are regularly consumed by different tribes in India, however, scanty information is available about the hazardous effects of most of these herbal products (Roy et al., 2009; 2010; Giri et al., 2013).

1.2 Geographical distribution and position of genus Carex  
The genus *Carex*, which consists of grasses and sedges, is the largest genus in the family Cyperaceae and contains more than 2000 species distributed throughout the Globe (Fiorentino et al., 2008a). Phyto-chemical constituents of the genus revealed to be different polyphenol and flavonoid (Kumar et al., 2013; Wisloff et al., 2003; Giovannini et al., 2001; Balint, 2000).

1.3 Traditional value of Carex baccans  
*Carex baccans*, locally known as “Kre”, is one such traditionally used medicinal plant of...
Northeast India, where indigenous tribal people use the crude aqueous juice of the roots to get rid of intestinal helminth infection (Challam et al., 2012). However, the plant is also used against cardiovascular, cerebrovascular diseases, hypertension, fever, dysmenorrhea, leucorrhea, chinchough, ulcer, meases, and fracture, as well as for gynaecological problems (He, 2012; Ghorbani et al., 2011; Zheng and Xing, 2009; Lee et al., 2008; Long and Li, 2004). Preliminary investigation carried out on albino rats, revealed that crude alcoholic extract of the plant causes hepatotoxicity in the animals (Roy et al., 2012).

2. Objective of Research

Because of public health importance of the plant in Northeast India and limited information available on its toxic effects on the consumers of the plant, the present in vivo investigation was, therefore, carried out to evaluate nephrotoxicity, if any, caused by the crude extract of the plant in wistar albino rat, through histomorphological, in situ cell death assay and ultrastructural observations.

3. Materials and Methods

3.1 Collection and processing of plant material

The root tubers of Carex baccans were collected from Meghalaya (25° 30’ N/ 91° 00’ E), India, during June – July, 2012, washed thoroughly, shade-dried and grounded by motor-driven grinder. 500g powder was then extracted with 90% methanol for 24 h at room temperature and the solution filtered through Whatman filter paper no. 1. The collected solution was dried through rotary evaporator and recovered 5g dry crude extract, which was stored at 4° C till further use.

3.2 Animals and treatment

Investigations on experimental animals were conducted in accordance with the institutions animal ethics committee guidelines for laboratory animal use and care. Adult Wistar rats 7-8 weeks old and weighing between 140-160 gm were obtained from Ghosh enterprises, Kolkata, and were kept in metal cages in the animal house having uniform temperature of 25° C with 12 h light and 12 h dark periodicity. The rats were fed with standard rat chows (Pranav agro feed Ltd., New Delhi, India) and water ad libitum. All animals were acclimatized for two weeks before starting the experiment. Subacute toxicity study was carried out in vivo using the sub-lethal doses. A total of eight rats of either sex (four males, four females) were selected for each set of experiment, out of the population of 40 by systematic randomization technique. The crude methanol extract of C. baccans administered once a day to the tested group by gastric feeding tube, at different doses like 40, 100, 200 and 400 mg kg⁻¹ body weight, dissolved in 1 ml of 0.9% normal saline having 0.1% (v/v) dimethylsulfoxide (DMSO) for 14 days. In case of control, rats were fed with normal saline having 0.1% (v/v) DMSO. Oral administration of extract was terminated on the day 14th, after which the rats were made to fast for overnight. On day 15th, the rats in each group were chloroform anesthetized and sacrificed for collection of kidney.

3.3 Tissue collection and processing for light microscopic study

On sacrificed, fresh kidney were recovered from extract exposed as well as control rats. After washing, kidneys were cut in to small pieces, fixed in Bouin’s solution and paraffin embedded prior to sectioning. Sections were cut at a thickness of 6 µm and stained with haematoxylin and eosin (H&E). Slides were viewed and photographed using compound microscope (Leica DM 2000, Germany).

3.4 Ultrastructural Studies

Organs of control and extract exposed animals were fixed in modified Karnovsky's fixative, post fixed in 1% OsO₄ buffered with 0.2 M sodium cacodylate for 4h, dehydrated through graded series of acetone and embedded in araldite. Ultrathin sections were stained with uranyl acetate, followed by lead citrate and viewed in a JEM 2100 (JEOL) transmission electron microscope, operated at 120 KV.

3.5 TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end lasbeling (TUNEL) method was used to detect apoptosis. Transverse sections of kidney were first fixed in 4% paraformaldehyde, exposed the sections with 10 mM Tris-HCL (pH: 8) and then incubated in proteinase K (20 µg/ml) for 15 min at room temperature. Sections were then incubated in elongation buffer. The fragmented DNA was labeled by biotinylated nucleotide mix in the presence of deoxynucleotidyl transferase (TdT) for 60 min in a humidified chamber and the reaction was stopped by transferring the slides to termination buffer (300mM NaCl, 30 mM Sodium citrate) for 15 min at room temperature. Then, the labeled fragments were incubated with extra avidin-peroxidase
and then stained with AEC (3-Amino-9-ethylcarbazole). Finally, the sections were counterstained with haematoxylin and viewed under light microscope (Leica DM 2000, Germany). From both the control and highest concentration plant extract exposed group, five slides were randomly selected and the apoptotic index (AI) was calculated as the percentage of positive cells, using the equation: \( AI = \frac{\text{number of positive cells}}{\text{total number of cells}} \times 100 \).

3.6 Nuclear condensation study by DAPI staining
At the end of experiment, kidney of the animal exposed to highest concentration of plant extracts (400 mg kg\(^{-1}\)) was taken out, washed and fixed in 4% paraformaldehyde. For the assessment of chromatin condensation and fragmentation, the sections were stained as described by Goping et al. (1999), using 1 µl/ml (Stock: 1 mg/ml) of DAPI (Himedia, Mumbai) in

sterile PBS at room temperature. The stained sections were examined under confocal microscope (Leica TCS SP5, Germany), using an excitation wavelength of 490 nm.

4. Results

4.1 Light microscopic observations
Light microscopic observations on the kidney of control rat (Fig. 1A) revealed normal features of Bowman’s capsule (BC), glomerulus (G), collecting tubule (CT) and distal tubule (DT). However, plant extracts administered rats revealed a distinct morphologic alteration as evidenced by glomerular damage, abnormal Bowman’s capsule, distal and collecting tubules (Fig 1B). Structural damage in the form of dilated cytoplasm (arrow head), damaged Bowman’s capsule shown by degeneration and dilation of sub-capsular space (thick arrows).

**Figure 1:** Photomicrographs of kidney of control (A) and extract of C. baccans exposed rat (B). A. Kidney showing normal Bowman’s capsule (BC), Glomerulus (G), collecting tubule (CT), distal tubule (DT) and compact arrangement cytoplasm. B. Kidney showing damaged Bowman’s capsule (BC), Glomerulus (G), dilated and degenerated sub-capsular space (thick arrows) with abnormal collecting (CT) and distal tubule (DT) with dilated cytoplasm (arrows head).

4.2 Ultrastructural observations
Transmission electron microscopic observation revealed normal architecture of basal infoldings of the distal tubules (arrows), which are common and continuous in control kidney (Fig. 2A). However, in the extract exposed group degenerated epithelial cells of distal tubule (DT) and expansion of the interstitial loose connective tissue were seen (Fig. 2B and 2C). Cortical distal nephron cells in the control group showed a normal nucleus, with nuclear membrane, mitochondria, compact cytoplasm with basal infoldings (Fig. 3A). In the control group glomerulus showing capillary loop (CL) were well preserved with filtration membrane (FM) (Fig. 3B). Normal glomerular filtration barrier (inset figure), revealed evenly distributed podocyte foot processes (white arrows) and uniform glomerular basement membrane (black arrows). Cells of kidney in the extract exposed animals were swollen, nucleus having small traces of nucleolus, deformed nuclear membrane (NM, arrow), distorted mitochondria of various size and shape, along with missing basal infoldings. Swollen mitochondria do not have clear cristae (Fig. 3C). Deformed capillary loop (CL), filtration membrane (FM), podocyte (P) and mesangial cell (MC) were observed in glomerulus (Fig. 3D). Inset figure showed disappearance of podocyte processes (thick black arrows) and discontinuous basement membrane (thin black arrow).
4.3 TUNEL assay
TUNEL assay showed very less or no apoptotic nuclei in control group (Fig. 4A). However, in the transverse sections of kidney of the plant extract exposed animals, clusters of dark brown spots were noticed, indicating in situ DNA fragmentation (Figs. 4B). In case of control and plant extract administered group AI were 1.38±0.889 and 11.38±1.072 respectively.

Figure 2: Transmission electron micrographs of control (A) and extract of C. baccans exposed kidney of rat (B and C). A. Enlarged view of control distal tubule (DT) showing continuous basal infolding (arrows); B. Kidney showing degenerating infolding in distal tubule (black arrows); C. Micrograph showing degenerating epithelial cells of distal tubule (DT) and expansion of the interstitial loose connective tissue (arrows)

4.4 Confocal Microscopy
Nuclei of control kidney tissue revealed to be compact in most of the cells when stained with DAPI. However, plant extracts exposed kidney tissue revealed fragmentation and condensation of chromatin, compared to the control (Fig. 5B).

4. Discussions
The principal functional unit of a kidney is the nephron, which consist of glomerulus, Bowman’s capsule, proximal and distal convoluted tubules. The glomerular filtration barrier consists of endothelium, glomerular basement membrane and the podocyte. Podocytes are contractile in nature and served as the final defense against urinary protein loss (Drenckhahn and Franke, 1988). Therefore, any structural alteration in the podocytes, as observed in our present study, leads to leakage of serum proteins in to the filtrate, resulting protein loss from the body (Whiteside and Dlugozy, 2002). Similar to our observations, crude extract of Narthecium ossifragum also causes deleterious effect on kidney of goat and rats (Wisloff et al., 2003; Balint, 2000). The plant Isotropis forrestii and Lilium asiatic has been reported to cause renal damage and/or toxicity in sheep and the damage was most prominent in the proximal convoluted tubules (Cooper et al., 1986; Brady and Janovitz, 2000). Likewise, in case of gentamicin-induced nephrotoxicity in lambs, patchy tubular necrosis recorded all through the cortex, but most prominent in the juxtamedullary area (Fadel and Larkin, 1996), which resulted in a reduction of number and size of endothelial fenestrate and thus a decrease in filtration pressure (Goldstein and Schnellmann, 1995).

Cellular swelling, disruption of renal epithelial cells, tubular dilation and lymphatic infiltration as observed in the nephron of rat in the present study was also recorded in mice exposed to arsenic trioxide subchronically at a dose 4 mg for 60 days (Li et al., 2010). Similar to the present observations, Alarifi et al. (2012) observed a marked glomerular and tubular alteration in the kidney of rat when exposed to gentamicin at 50 mgkg-1 body weight. Tubular necrosis and glomerular destruction by the drug was also observed in the kidney of agouti which mimic human physiology (Cabral et al., 2012).
**Figure 3:** Transmission electron micrographs of control (A and B) and extract of *C. baccans* exposed kidney of rat (C and D). **A.** Section showing epithelial cell having well preserved nucleus, nuclear membrane and plenty of normal mitochondria (M, stars); **B.** Section of a portion of Bowman’s capsule showing normal capillary loop (CL) and filtration membrane (FM). Inset figure showing well-arranged podocyte processes (white arrows) with intact glomerular basement membrane (black arrow), enlarged from white encircled portion; **C.** Section showing grossly affected mitochondria (M, stars), vacuolated nucleus, disrupted and disintegrated (arrows) nuclear membrane (NM); **D.** Section showing notable changes in the podocytes (P), filtration membrane (FM), mesangial cells (MC) and capillary loop (CL) of Bowman’s capsule. Inset figure showing disappearance of podocyte processes (thick black arrows) and discontinuous basement membrane (thin black arrows), enlarged from white encircled portion.

**Figure 4:** Light microscopic photograph of a section of kidney showing control (A) and TUNEL stained apoptotic cells (arrow) (B).

**Figure 5:** Confocal microscopic photograph of control (A) and extract of *C. baccans* treated (B) kidney of rat. **A.** Section showing compact nucleus with less or no condensation; **B.** Section showing chromatin condensation and fragmentation (arrows).
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Extensive damage in the proximal tubule as observed in our present study could be due to the fact that the proximal convoluted tubules are the primary site of reabsorption and active transport resulting higher accumulation of phytochemical in the epithelial lining of these tubules. Recently, Kang et al., (2011) observed that 4-hydroxy-2-nonal which is found plenty in food product increase kidney weight at a dose 12.5 mg/kg per day for 28 days and damage the kidney through accumulation of hyaline bodies in the renal tubule leading to degeneration of tubular epithelium.

Cellular changes like disruption of mitochondria, condensation of nucleus, disintegration of nuclear membrane as observed in the present study are indication of apoptotic cell death caused by the plant C. baccans, which was confirmed through observations of fragmented DNA by TUNEL assay. The quantitative expression of apoptotic nuclei in plant extract exposed groups was significantly high compared to the control. Several workers showed that phytochemical induced apoptosis followed the intrinsic pathway, where as the drug/toxin damage the mitochondria through altering mitochondria membrane potential. Cytochrome C than release from mitochondria to the cytosol and interact with apoptotic protease leading to cell death (Zhang et al., 2011; Karunagaran et al., 2005; kromer and Reed, 2000). (+)-α-viniferin, an active phyto stilbene, found in several species of the genus Carex, revealed to be proapoptotic to several cancer cell lines (Chowdhury et al., 2005), apart for having antioxidant (Jin et al., 2012) and anti neurotransmitter properties (Sung et al., 2002). However, González-Sarroías et al. (2011) observed that the phytochemical did not induce apoptosis in vitro, but arrest cell cycle in colon cancer cells.

The nephrotoxic principle in C. baccans was shown to be fast acting, as ultrastructural changes were observed in glomeruli, tubules, and vascular endothelium both in cellular and organelles level. Plant extracts induced confirmatory apoptosis study also further support the outcomes. However, detail molecular approach involving active principle(s) of the plant for elucidating the possible mechanism of apoptotic cell death pertaining to nephrotoxicity is pre requisite to draw a concrete conclusion.

Author’s Contribution and Competing Interests:
BR and BRG designed the study; BRG conducted the experiment; BR and BRG analyzed the data and wrote the paper. The authors declare no competing interests.

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