Antiproliferative and DNA-cleaving Activities and Phytochemical Screening of *Balanites roxburghiana* Linn. Fruit Extracts

S. P. SOUL SHEKHAR, K. MANJULATHA[1], N. D. SATYANARAYAN* AND W. A. AL-BAADANI

Department of Pharmaceutical Chemistry, Kuvempu University, Postgraduate Centre, Kadur, Chikmagalur-577 548, [1]School of Life Science, University of Hyderabad, C. R. Rao Road, Gachibowli, Hyderabad-500046, India

The present investigation dealt with the antiproliferative, DNA-cleaving and preliminary phytochemical screening of n-hexane, dichloromethane and methanol extracts of *Balanites roxburghiana* Linn. The 3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide assay was used to assess the antiproliferative activity against chronic myelogenous leukaemia, hepatocellular carcinoma, breast cancer, cervical cancer, colorectal adenocarcinoma and normal human kidney embryonic cell lines. Calf-thymus DNA-cleavage analysis was performed by agarose gel electrophoresis at various concentrations from 10 to 100 µg and preliminary phytochemical screening has been performed using standard procedure. The antiproliferative assay results of the extracts revealed that the n-hexane and dichloromethane extracts were more active compared to the methanol extract. The methanol extract showed 21.7 % activity against breast cancer cell lines only. n-Hexane extract (V_1) exhibited DNA cleavage at all concentrations, which could be due to binding to DNA. The dichloromethane extract (V_2) exhibited DNA cleavage at all concentrations but a very prominent low molecular weight DNA band formation could be observed with increasing concentration of the extract, With the methanol extract (V_3), the DNA cleavage increased with concentration of the sample. A partial cleavage could be found at 10 µg concentration under a UV transilluminator. The preliminary phytochemical screening revealed presence of flavonoids, steroids, tannins and saponins.

Key words: *Balanites roxburghiana*, antiproliferative, DNA cleavage, phytochemical screening, agarose gel electrophoresis, MTT assay, UV transilluminator

Cancer is one of the most severe disease in which unregulated proliferation of abnormal cells invade and disrupt surrounding tissues[1]. It begins in the cells of the body, where the orderly process is disturbed by production of unwanted new cells without the lysis of old cells thus these extra cells lump together to form a growing tumor[2]. It is the leading cause of morbidity and mortality worldwide with approximately 14.1 million new cases, 8.2 million cancer related deaths and is expected to rise by 70 % over the next two decades as reported by GLOBOCAN (Global cancer) 2012 and the International Agency for Research on Cancer online database[3]. The partially successful clinical therapies include radiation, chemotherapy and surgery, indicating an urgent need of alternative strategies[4]. Medicinal plants have been used as a remedy for treating of human diseases for centuries, because of the presence of secondary metabolites of therapeutic value[5] such as alkaloids, flavonoids, tannins and phenolics[6], which are expected to play a vital role in the treatment of cancer[7]. The majority of these compounds are capable of scavenging free radicals. The major chronic health problems such as cancer, heart diseases, inflammation, neurodegeneration, aging and also food deterioration are due to oxidative stress[8]. For offering the required protection to avoid oxidative stress and related permanent alterations of biomolecules, *in vitro* and *in vivo* studies have shown the effective role of phenolics in the preclusion or inhibition of disorders such as oxidative damage to DNA, proteins and lipid, or many chronic diseases[9]. Flavonoids may act through inhibiting cytoplasmic membrane function and also by

***Address for correspondence**
E-mail: satya1782005@gmail.com

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inhibiting of DNA gyrase and beta-hydroxyacyl-acyl carrier protein dehydratase activities[10]. The isoflavone, genistein was able to modify cell morphology by formation of filamentous cells and inhibited the synthesis of DNA and RNA of *Vibrio harveyi*[11]. Plant secondary metabolites could maintain the health and cure various diseases including cancer with less harm full effects[12]. *Balanites roxburghiana* Del (family: Zygophyllaceae), known as desert date in English is found abundantly in dry lands of Africa and South Asia and is most common but neglected wild species[13]. It is a multi-branched, thorny shrub or tree grows up to l0 m tall. Crown is spherical in one or more distinct masses. Trunk is short and often branching from near the base. Bark is dark brown to grey and completely fissured. Branches are armed with yellow or green thorns up to 8 cm long. Leaves are with two separate leaflets; leaflets are obovate, asymmetric, 2.5 to 6 cm long, bright green, leathery, with fine hairs when young. Flowers are fragrant, yellowish green growing in fascicles of the leaf axils. Fruit is rather long, narrow drupe, 2.5 to 7 cm long, 1.5 to 4 cm in diameter. Immature fruits are green and tormentose, turning yellow and glabrous when mature. Pulp of the fruit is bitter-sweet to taste and edible. Seed is the pyrene (stone), measures as 1.5 to 3 cm long, light brown, fibrous, and tremendously hard[14]. This plant exhibited different pharmacological activities such as cardioprotective, antioxidant[15], anthelmintic[16], antibacterial[17], antivenin[18], antiinflammatory, analgesic[19], antioxidant, xanthine oxidase and acetyl cholinesterase inhibitory[20], antinociceptive, antioxidant[21], mosquito larvicidal[22], hepatoprotective[23], antiviral[24], wound healing[25], hypocholesterolemia[26], diuretic[27], aldose reductase inhibitory[28] and antidiabetic[29]. Compounds isolated from this plant such as balanitin-6,7-saponin[30] and steroidal saponin[31] have shown anticancer activity. Fruit extract has inhibited proliferation of Ehrlich ascitic tumor[32] and methanol extract of fruit has shown antiproliferative activity against breast, colon, and liver cancer cells in a sulforhodamine B assay[33]. The above documented literature revealed that *B. roxburghiana* is a medicinally important plant and has to be considered for detailed study to understand its mechanism of action against cancer and also to check against other cell lines, which are not previously investigated. Hence, the present study was aimed to determine antiproliferative activity along with DNA-cleaving activity using 3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay and agarose gel electrophoresis of various solvent extracts of *B. roxburghiana* fruit, respectively.

The chemicals used were of analytical grade. Doxorubicin (Sigma-Aldrich, USA), n-hexane, dichloromethane, methanol, dimethyl sulfoxide (DMSO; HiMedia), calf-thymus DNA (Genei, Bengaluru) other chemicals such as agarose gel and ethylene bromide used for the study were also purchased from (HiMedia). The fruits of *B. roxburghiana* were collected in the month of February to March-2014 around Kadur town of Chikmagalur, India and were authenticated at the Herbarium, Department of Botany, Kuvempu University, Shankaraghatta, Shimoga, India. The collected fruits were immediately sprayed with alcohol to cease enzymatic degradation of secondary metabolites. The fruits were stored in cool, dry place before extraction.

The shade-dried fruits (79.3 g) of *B. roxburghiana* were chopped into small fragments of 1-2 inches in length and extracted with different solvents *viz*. n-hexane, dichloromethane and methanol successively in a Soxhlet extractor for about 72 h each. The solvent was evaporated under reduced pressure and controlled-temperature using a Buchi evaporator. The solvent-evaporated mass of n-hexane, dichloromethane and methanol extracts were 0.68, 0.76 and 10.52 g, respectively. The extracts were stored in a freezer (–4°) until further use. Preliminary phytochemical screening of *B. roxburghiana* fruit was performed using standard procedures[34,35]. The results were shown in Table 1.

Six different cell lines, colorectal adenocarcinoma (COLO 205); chronic myelogenous leukaemia (K562); breast adenocarcinoma (MCF 7); cervical cancer (HeLa); normal human kidney embryonic cell line (HEK293) and hepatocellular carcinoma (HepG2) were used in this investigation. All cell lines were obtained from the National Centre for Cell Sciences, Pune, India, and were cultured at a seeding density of 0.2×106 in Dulbecco's modified Eagles medium/Roswell Park Memorial Institute (DMEM/RPMI) medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, respectively maintained in a humidified atmosphere with 5 % CO_2 at 37°. The samples were dissolved in DMSO (not exceeding the final concentration of 0.01 %) and further diluted in cell culture medium. The antiproliferative response of extract was assessed using the MTT assay[36]. Cells (~10 000) were plated in 200 µl growth medium in the presence or absence of the extract (25, 50, 100, and

200 µg/ml) in 96-well culture plates for 24 h. Then the culture plates were centrifuged at 2000 rpm for 10 min at room temperature. Supernatant (100 µl) was discarded and 20 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°. The viability of the cells was determined at 570 nm using a spectrophotometer.

The extract was added separately to the DNA sample. The sample mixtures were incubated at 37° for 2 h. The treatment of DNA samples and the electrophoresis of these samples were performed according to the following procedure[37]. Two hundred and fifty milligrams of agarose was dissolved in 25 ml of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1: l) with boiling. As the gel attained ~55°, it was poured into the gel cassette fitted with comb and the gel was allowed to solidify. The comb was carefully removed and the gel was placed in the electrophoresis chamber flooded with TAE buffer. DNA sample (20 µl) mixed with bromophenol blue dye at 1:1 ratio was carefully loaded into the wells along with DNA marker and a constant 50 V of electricity was passed for around 45 min. The gel was removed carefully, stained with ethydium bromide solution (10 µg/ml) for 10-15 min and the bands were observed under a UV transilluminator. The preliminary phytochemical screening of the n-hexane, dichloromethane and methanol extracts of the fruits of *B. roxburghiana* revealed the presence of saponins, tannins, steroids and flavonoids.

The result for antiproliferative activity by different extracts of *B. roxburghiana* fruit on different cell lines were shown in Table 2. The ongoing research is to seek out effective treatments for cancer including the use of medicinal plants. This treatment makes use of the compounds naturally present in plants that are known to inhibit or kill carcinogenic cells[38]. Phytochemical screening of the fruit extracts showed the presence of flavonoids, steroids, tannins and saponins (Table 1). Flavonoids have been reported to inhibit xanthine oxidase[39] and cyclooxygenase[40] enzymes. The molecular mechanism might involve the inhibition of the pro-oxidant process that causes tumor promotion. Growth promoting oxidants and reactive oxygen species are the major catalysts of the tumor promotion and progression stages and therefore antioxidants inhibit tumor cell proliferation. In addition, the mechanism of inhibition of polyamine biosynthesis can contribute to the antiproliferative activities of flavonoids. Ornithine decarboxylase is a rate-limiting enzyme in polyamine biosynthesis and is correlated with the rate of DNA synthesis and cell proliferation in several tissues.

TABLE 1: SHOWING THE RESULT OF PRELIMINARY PHYTOCHEMICAL SCREENING OF *B. ROXBURGHIANA* FRUIT EXTRACT

Test	Phytochemical test	BRH	BRD	BRM
Alkaloids	Mayer's test	-	-	-
	Wagner's test	-	-	-
Flavonoids	Ferric chloride test	-	-	+
	Alkaline test	-	+	+
Glycosides	Killer-Killan's test	-	-	-
	Bromine water test	-	-	-
Steroids	Salkowaski's test	+	+	+
	Liebermann Burchad test	+	+	+
Tannins	Ferric chloride test	-	-	+
Saponins	Foam test	+	+	+
Carbohydrates	Molisch's test	-	-	+
	Benedicts test	-	-	-
Proteins	Xanthoprotein test	-	-	-
	Ninhydrin test	-	-	-

BRH- *B. roxburghiana* n-hexane extract, BRD- *B. roxburghiana* dichloromethane extract and BRM- *B. roxburghiana* methanol extract

TABLE 2: ANTIPROLIFERATIVE ACTIVITY OF SOLVENT EXTRACTS OF *B. ROXBURGHIANA* FRUIT BY MTT ASSAY ON WITH DIFFERENT CANCER CELL LINES

Cancer cell lines	Average inhibition	BRH	BRD	BRM	Doxorubicin concn 1uM
K562	Average inhibition (%)	9.99763	12.9898	11.5887	95.57
	SD	3.6373	2.17129	2.17876	2.2256
MCF – 7	Average inhibition (%)	11.624	20.1765	21.7037	97.61
	SD	1.59556	2.09651	2.73341	2.189
HeLa	Average inhibition (%)	13.5706	13.2369	12.6808	97.16
	SD	1.97422	2.22469	3.33519	2.27
COLO	Average inhibition (%)	21.7153	10.4015	11.2226	91.55
	SD	3.50687	1.21466	3.61641	1.8723
HepG2	Average inhibition (%)	9.18919	15.9214	16.6585	97.355
	SD	2.82746	2.57552	2.08484	1.5699
HEK	Average inhibition (%)	-1.55109	0.44668	-6.48649	5.678
	SD	3.1856	1.61342	3.22495	1.56

BRH- *B. roxburghiana* n-hexane extract, BRD- *B. roxburghiana* dichloromethane extract and BRM- *B. roxburghiana* methanol extract

Several studies reported that flavonoids could inhibit ornithine decarboxylase induced by tumor promoters causing a subsequent decrease in polyamine and inhibition of DNA and protein synthesis[41]. Steroids might bind to Na^+/K^+-ATPase resulting in a complex but well-documented changes in cell signalling events. The signalosome complex included the enzyme, Na^+/K^+-ATPase as well as, phosphoinositide-3 kinase and phospholipase each of which, in turn, set into action complex signalling events that resulted in tumor cell death through either apoptosis or autophagy-related mechanisms[42].

The antiproliferative and DNA-cleaving potential of various extracts of *B. roxburghiana* fruit have shown less significant activity against different cell lines but methanol and dichloromethane extracts have shown significant activity against MCF-7 cell line and n-hexane against co COLO 205 cell lines. From the above result, it could be predicted that the inhibition of MCF-7 cells by methanol and dichloromethane extract would be most effective and the inhibition of COLO 205 cell line by n-hexane extract is the best among the extracts screened.

The results obtained have shown that the three extracts of *B. roxburghiana* fruit has moderate inhibition on different cancer cell lines. The evaluation is carried out by dissolving the extracts in DMSO (not exceeding the final concentration of 0.01 %) and further diluted in cell culture medium. The antiproliferative response of the extract was assessed by MTT assay using doxorubicin as a standard drug molecule. The viability of the cells was determined using a spectrophotometer at 570 nm. The IC_{50}, that is, the concentration of the extract required to inhibit cell growth by 50 %, was determined.

DNA-cleaving activity of n-hexane, dichloromethane and methanol extracts of *B. roxburghiana* fruit was studied using agarose gel electrophoresis method and the results were presented in fig. 1. DNA was the target for drug discovery as it regulated many biochemical reactions. Clinical efficacies of many drugs can be correlated with their ability to induce enzyme-mediated DNA-cleavage. The loci present in DNA are involved in regulatory processes such as gene expression, gene transcription, mutagenesis and carcinogenesis[43]. In particular, designing of the compound having ability to cleave DNA is utmost important not only from the primary biological point of view but also in terms of photodynamic therapeutic approach to develop potent drugs[44]. The extracts, which were found to be active in DNA-cleavage assay, were further screened for antiproliferative study. DNA-cleaving potential of the extracts were examined by comparing the band appeared in control and test samples at 100 µg concentration, fig. 1 clearly demonstrated that with n-hexane extract (V_1), DNA cleavage could be seen at all concentrations, with dichloromethane extract (V_2), DNA cleavage was observed at all concentrations but a very prominent low molecular weight DNA band formation could be seen with increasing concentration of sample and with methanol extract (V_3), DNA cleavage increased with the concentration of sample. A partial cleavage could be found at 10 µg and cleavage activity increased along with sample concentrations.

The current study is to assess the preliminary phytochemical screening, DNA cleavage and antiproliferative activity of various extract of *B. roxburghiana* fruit, which showed the presence of flavonoids, steroids, tannins and saponins. Out of these

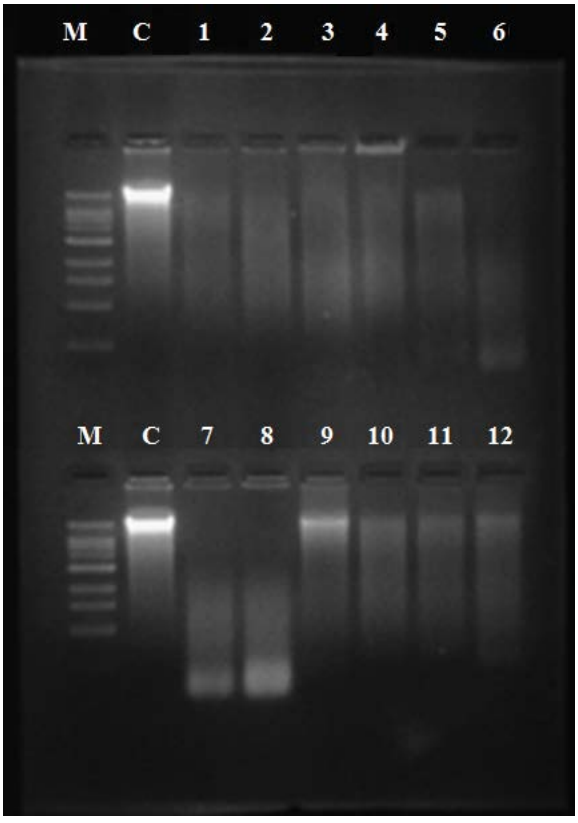

Fig. 1: Gel picture showing the cleavage analysis of n-hexane, dichloromethane and methanol extracts of *B. roxburghiana* fruit

M- Supermix DNA Ladder (Merck, Cat # MBD21J); C-CT-DNA; 1, 2, 3, 4- sample V_1 at 10, 25, 50 and 100 µg, respectively; 5, 6, 7, 8- sample V_2 at 10, 25, 50 and 100 µg, respectively; 9, 10, 11, 12- sample V_3 at 10, 25, 50 and 100 µg, respectively. V_1, V_2 and V_3 are n-hexane, dichloromethane and methanol extracts of *B. roxburghiana* fruit

secondary metabolites some are responsible for DNA cleavage and antiproliferative activity. DNA cleavage could be seen at all concentrations of the n-hexane extract 10, 25, 50 and 100 µg, the intensity of DNA attached to the wells was observed more. This might be because of sample binding to DNA, dichloromethane extract showed very prominent low molecular weight DNA band formation and methanol extract has shown a partial cleavage at 10 µg concentration. The results revealed that the n-hexane and dichloromethane extracts were the most active compared to the methanol extract. The methanol extract has shown activity only against breast cancer cell line. Thus *B. roxburghiana* fruit appeared to be significantly active against cancer cell lines in the MTT assay and active in the DNA-cleavage agarose gel electrophoresis assay. Further research is needed to isolate specific phytochemicals from the extracts that were responsible for the antiproliferative activity against MCF-7 and COLO 205 cell lines.

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There are no conflicts of interest.

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