INNOVARE JOURNAL OF LIFE SCIENCE

Vol 7, Issue 2, 2019

ISSN - 2321-550X

Research Article

RADIOMIMETIC DRUG – "BLEOMYCIN-" INDUCED DNA DAMAGE REPAIR BY *ALSTONIA SCHOLARIS* BARK EXTRACTS – A G2 ASSAY-BASED EVIDENCE

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Received:11 January 2019 Revised and Accepted: 26 March 2019

ABSTRACT

Objective: The present study initiated to know the probable DNA damage repair by *Alstonia scholaris* extracts using bleomycin-induced chemosensitive G2 assay in *in vitro* cultured human blood lymphocytes.

Methods: The plant extracts were from bark, stem, and leaves and extraction solvents used were double-distilled water and methanol. The experiments were carried out with blood samples collected from 12 healthy volunteers. A total of 28 culture vials from each individual were set up for lymphocyte cultures. These culture tubes (vials) were divided into two groups of fourteen each. The first group was labeled as "G0," while the second as "G2." In both the groups, initial vials ("A") were kept untreated so as to act as a control, the second vial labeled ("B") received treatment of alone dose of BLM (15 µg/ml), while vials labeled "C"–"N" were treated with varied combinations. At the 71st h, the cells were treated with 100 µl of 0.001% colchicine. The cultures were terminated at the 72th h of incubation. Routine air-dried preparations were made. Each slide was blind coded, conveniently stained in 2% Giemsa, and scored under ×100 oil immersion lens.

Results: The results indicated that aqueous as well as methanolic bark extracts had enough potential to repair DNA damage caused by bleomycin. The extracts (both aqueous and methanolic) from stem or leaves were not promising.

Keywords: DNA damage repair, *Alstonia scholaris,* Bleomycin, Human lymphocyte cultures, G2 assay.

INTRODUCTION

Alstonia scholaris Linn. (Family: Apocynaceae) is commonly known as the "Saptaparni" or "Devil's tree" in India. It is widely distributed in drier forests, Western Himalayas, Western Ghats, and Southern region of India. It is a well-known remedy for the treatment of various types of disorders in the Ayurvedic, Homoeopathic, and Folklore system of medicine [1,2].

Alkaloids are one of the major constituents of *Alstonia* species [3-12]. Over 70 different types of alkaloids have been reported in different parts of *A. scholaris* such as root, stem bark, leaves, fruit, and flower [13]. Among other constituents, isookanin-7-o-alpha-lrhamnopyranoside, flavanone glycoside [14] and alstonoside, and secoiridoid glucoside [15] have been recorded. Iridoids, coumarins, flavonoids, leucoanthocyanins, reducing sugars, simple phenolics, steroids, saponins, and tannins were also found in the *A. scholaris* [16]. Presence of agr-amyrin, bgr-amyrin, lupeol acetate, venenative, rhazine, and yohimbine has been noted [17]. Linalool, cis- and trans-linalool oxides (furanoid and pyranoid), alpha-terpineol, 2-phenylethyl acetate and terpinen-4-ol [18], and steroids [19] have also been reported as phytoconstituents of the *Alstonia* sp.

In India, the therapeutic use of *A. scholaris* has been described in both codified and non-codified drug systems for the treatment of malaria, jaundice, gastrointestinal troubles, and cancer and in many other ailments [13]. Other species, *Alstonia macrophylla* has been used in conventional medicines in Thailand, Malaysia, and Philippines as a general tonic, aphrodisiac, anticholeric, antidysentery, antipyretic, emmenagogue, and vulnerary agents [1,20-24]. At times, *A. macrophylla* is also used as a substitute for *A. scholaris* in various herbal pharmaceutical preparations.

Several biological and pharmacological studies were undertaken to evaluate traditional and ethnopharmacological claims of *A. scholaris*. Thus, so far, various biological and pharmacological activities such as antimalarial [25,26] antimicrobial [27-30], antidiarrheal [31], antioxidant [32-35], antidiabetic [36], anticancer and cytotoxicity [37-39], analgesic [40], anti-inflammatory [41], hepatoprotective and central nervous system [42-43], wound healing [44], immunostimulating [45,46], antitussive and antiasthmatic [47], and antifertility [17] properties have been reported to *A. scholaris.*

Gupta *et al.* [48] reported radioprotective efficacy of bark extracts of *A. scholaris* in mice against radiation-induced hematological (total number of erythrocytes, percentage of hematocrit, and hemoglobin) and biochemical alterations (lipid peroxidation and glutathione content). The bark extract of *A. scholaris* was found to restore the total leukocytes and differential leukocytes (lymphocytes, monocytes, neutrophils, and non-neutrophilic granulocytes) count in *A. scholaris* bark extract pre-treated animals as compared to the irradiated control group. Another study [49] from the same laboratory reported that radiation-induced augmentation in lipid peroxidation and cholesterol level was significantly ameliorated in mice fed with *A. scholaris* bark extract. Jahan and Goyal [50] demonstrated that *A. scholaris* bark extract pre-treatment in mice provides protection against radiation-induced cytogenetic damage in the form of chromosomal aberrations and micronuclei induction in bone marrow cells.

From the foregoing discussion, we hypothesized the applicability of *A. scholaris* (L.) R. Br. as a plant with protective property against chemical-induced cytogenetic damage. The present study was, therefore, initiated to know the probable DNA damage repair by *A. scholaris* extracts using bleomycin-induced chemosensitive G2 assay in *in vitro* cultured human blood lymphocytes.

METHODS

The bark, stem, and leaves of *A. scholaris* were collected separately and dried for 2–3 days at room temperature. After drying, they were ground crumbly. The finely ground powder was placed in a porous bag (thimble) made of strong filter paper. The thimble was placed in the

chamber of the Soxhlet apparatus. Two separate solvents (methanol and double-distilled water) were used for extraction. The extraction assembly was set up as described [51]. Hot continuous extraction was carried out. After 5–6 refluxing cycles, a drop of solvent from the siphon tube was collected and observed for residue mark after evaporation. When no residue was found from the evaporated drop so collected, the refluxing cycle was terminated. The solvent containing extract siphoned out into the distillation still was collected and allowed to cool. The liquid content was evaporated and condensed. This was stored in sterile labeled container at 4°–8°C until further use. Extracts were redissolved in sterile pyrogen-free water and sterilized using syringe filters before use.

G2 assay

The experiments were carried out with blood samples collected from 12 healthy volunteers. A total of 28 culture vials from each individual were set up for lymphocyte cultures [52]. These culture tubes (vials) were divided into two groups of fourteen each. The first group was labeled as "G0," while the second as "G2." In both groups, initial vials ("A") were kept untreated so as to act as a control, the second vial labeled ("B") received treatment of alone dose of BLM (15 μg/ml), while vials labeled "C" to "N" were treated as shown in Table 1. At the 71st h, the cells were treated with 100 μl of 0.001% colchicine. The cultures were terminated at the 72th h of incubation. Routine air-dried preparations were made. Each slide was blind coded, conveniently stained in 2% Giemsa, and scored under ×100 oil immersion lens. To avoid observer's bias, all slides were scored twice by different observers. The average of total chromatid aberrations recorded by each observer has been tabulated.

RESULTS AND DISCUSSION

The results of G2 assay revealed a statistically significant ($p<0.05$) reduction in the total chromatid breaks from control cultures [52] belonging to "G2" group as compared to control cultures (71) belonging to "G0" group (Table 2), indicating that DNA repair mechanisms were functional in all blood donors studied.

The total chromatid breaks were significantly less in cultures belonging to "G2" group as compared to respective cultures belonging to "G0" group for all groups studied. However, there was variation in the level of significance with each treatment condition. The highest level ($p<0.0001$) of reduction in total chromatid breaks was observed from those cultures treated with aqueous bark extracts at G2 phase (22) than those at G0 phase (60). No significant reduction was observed when G2 tubes treated with stem and leaf extracts were compared to respective tubes of G0 group.

Devastating effects of radiation pose the need for radioprotectors to safeguard different organs of our body and to avoid the lethality associated with these radiations. Radioprotector is a group of measures, designed to ensure man and his environment are protected against the harmful effect of ionizing radiations. They are effective to save our bodies from wanted or unwanted radiations. Hazardous radiations cause consequential injuries to biological systems; therefore, it is a necessity to formulate such pharmacologically dynamic radioprotector that can render protection to humans against destructive and damaging outcome of ionizing radiation. Cellular adaptations and mechanisms to counteract the lethal consequences of damage by radiation have been well studied [53]. Radioprotectors ensure the elevation of non-protein sulfhydryl groups, reduction in lipid peroxidation, and upregulation of free radical scavenging activity through transcription upregulation of antioxidant enzymes such as glutathione transferase, catalase, superoxide dismutase, and glutathione peroxidase. Radiation caused damage can also be neutralized by the upregulation of DNA repair activity. Other mechanisms, which help in radioprotection, are the inactivation of protein kinase (PK)-C, nitric oxide, mitogen-activated PK, and downregulation of several other effectors responsible for molecular damage.

Even though many substances with assumed cytoprotective effects are the subjects of laboratory and/or clinical studies, at the moment, there is no ideal protective strategy to be universally employed in patients receiving radio- or chemotherapy [54]. It has been established during the last two decades that products derived from natural sources could be used as non-toxic radioprotectants.

Majority of the plants are either radioprotectors or radiomodulators, but plants with potency to repair the damage caused by radiation,

Table 1: Treatment protocol for G2 assay

Group	Vials	Treatment at 0 h	Treatment at 70 h
G0	A	Control	Nil
	B	Bleomycin (15 μg/ml)	
	C	Aqueous bark extract (50 μg/ml)	
	D	Methanolic bark extract (50 μg/ml)	
	E	Aqueous stem extract (50 μg/ml)	
	F	Methanolic stem extract (50 μg/ml)	
	G	Aqueous leaf extract (50 μg/ml)	
	H	Methanolic leaf extract (50 μg/ml)	
	I	Aqueous bark extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	J	Methanolic bark extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	K	Aqueous stem extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	L	Methanolic stem extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	M	Aqueous leaf extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	N	Methanolic leaf extract (50 μg/ml) + Bleomycin (15 μg/ml)	
G2	A	Control	Bleomycin (5 μg/ml)
	B	Bleomycin (15 μg/ml)	
	C	Aqueous bark extract (50 μg/ml)	
	D	Methanolic bark extract (50 μg/ml)	
	E	Aqueous stem extract (50 μg/ml)	
	F	Methanolic stem extract (50 μg/ml)	
	G	Aqueous leaf extract (50 μg/ml)	
	H	Methanolic leaf extract (50 μg/ml)	
	I	Aqueous bark extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	J	Methanolic bark extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	K	Aqueous stem extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	L	Methanolic stem extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	M	Aqueous leaf extract (50 μg/ml) + Bleomycin (15 μg/ml)	
	N	Methanolic leaf extract (50 μg/ml) + Bleomycin (15 μg/ml)	

Table 2: Comparison between G0 and G2 groups of total chromatid breaks induced in *in vitro* cultured human lymphocytes after addition of aqueous and methanolic extracts from bark, stem, and leaf of *Alstonia scholaris* alone as well as with bleomycin (values indicate aberrations per 1200 cells)

Groups	Vials													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Total chromatid break														
G0	71	254	60	39	66	53	68	62	76	84	73	69	77	92
G2	52	204	22	24	48	44	62	51	47	65	66	61	69	86
p values (ANOVA analysis)	0.0420	0.0346	0.0001	0.0420					0.0030	0.0342				

i.e., radio-mitigators have been rarely reported. It is very likely that no single mechanism can account for the protection offered by a radioprotective drug [55]. Radiation is known to produce oxygen-free radicals which are implicated in the process of DNA damage, cell killing, mutagenesis, and carcinogenesis [56]; hence, it is reasonable to assume that agents capable of scavenging free radicals would play a significant role in modulating these processes. The radioprotection of normal cells by a number of synthetic and natural compounds has been reported to be mediated through free radical scavenging activity [56].

Different radioprotectants offer protection to cellular molecules by different mechanisms [57]. Some of these compounds protect the target molecules due to their antioxidant potentials by neutralizing the free radicals, others enhance the cellular DNA repair, certain modifies the signaling pathways, some modulate the immune system, and few contribute to a combination of above-mentioned mechanisms [58,59].

The bark of *A. scholaris* is the most intensively used part of the plant and is used in many compound herbal formulas [60]. In the present study, the qualitative and quantitative analysis of extracts from the bark of *A. scholaris* revealed the presence of flavonoids. In addition, the quantity of terpenoids was highest in aqueous extracts as compared to other compounds followed by steroids, alkaloids, and phenolic compounds.

It has been reported that plant secondary metabolites such as flavonoids and terpenoids play an important role in defense against free radicals [58]. The free radical scavenging and antioxidant potential of *A. scholaris* observed in the present finding could be attributed to terpenoids, steroids, alkaloids, flavonoids, and phenolic compounds. Thus, antioxidant and free radical scavenging potential of the plant so exhibited could be responsible for radioprotective potentials. Plants that produce antioxidants which scavenge free radicals caused by radiation exhibit radioprotective action.

The results of our present study add to this body of knowledge one more plant (*A. scholaris*) with such potentials. The results of our chemosensitive G2 assay clearly suggest that certain compound/s could be present in aqueous bark extracts which enhance DNA repair capacity. Owing to this fact the therapeutic value index of the plant *A. scholaris* seems higher, since it is more logical to treat after the damage has occurred.

So far, the explanation for radioprotective or radiomodulatory action of *A. scholaris* has been attributed to its antioxidant potentials and free radical scavenging activity [48,50,61-65]. The key aspects of radiation protective agents are the practical need to use them in specific scenarios of radiation exposure and the corresponding tactical and technical requirements for medical preparations [60]. Radioprotectors realize radiation protective action at the cellular level in the course of rapidly proceeding radiation-chemical reactions. At the same time, when the ionizing radiation energy is absorbed, these agents partially neutralize the "oxygen effect" as a radiobiological phenomenon, especially in the radiolysis of DNA [66].

Certain group of agents used in medicine exerts their effect on system levels by promoting the acceleration of the post-radiation restoration of radiosensitive tissues and is not directly connected with the primary radiation chemical and biochemical processes in cells that occur during the absorption of the energy from ionizing radiation. For this reason, these agents are effective not only at administration before irradiation but also during the early period after, acting as mitigators [60]. Radiomitigators exert their effect on system levels by promoting the acceleration of the post-radiation restoration of radiosensitive tissues through an activation of pro-inflammatory signaling pathways and a stimulation of hematopoietic stem and progenitor cells and mesenchymal stromal cell mobilization [60].

DNA repair is a ubiquitous mechanism that is critical to maintain the integrity of the genome [60]. Few plants (*Mentha piperita* and *Mentha arvensis* [68,69], *Grewia asiatica* [70,71], and *Arabidopsis* [72]) have enhanced radiation-induced DNA damage repair processes. Our finding adds to this body of information one more plant (*A. scholaris*) with such mitigatory action. To the best of our knowledge, this study is the first of its kind, where radiomitigatory activity of the said plant has been reported using cultured human lymphocytes. These data have important application for the protection of human lymphocytes from the genetic damage and side effects induced by X-ray irradiation in patients undergoing radiotherapy. However, an extended examination of actual and individual phytochemicals from the plant responsible for radioprotection, radiomodulation, and radiomitigation would help better understand the probable mechanisms and increase the applicability value of the plant.

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