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In vitro Antiproliferative and inhibition of oxidative DNA damage activities of n-butanol extract of Limonium bonduelli from Algeria

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HIGHLIGHTS

- *L. bonduelli* showed high phenolic and flavonoid contents
- It showed a broad-spectrum antioxidant properties including DNA protection
- It showed concentration-dependent antiproliferative effects
- *L. bonduelli* is a rich source of natural antioxidants and anticancer agents

Abstract: Plants are the main sources of natural antioxidants in the form of phenolic compounds, which help human beings to deal with oxidative stress, caused by free radical damage. For this reason, the present study was carried out to evaluate the antiproliferative, antioxidant and inhibition of oxidative DNA damage activities of *n*-butanol extract obtained from aerial parts of *Limonium bonduelli*. The antioxidant potential was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and inhibition of lipid peroxidation assay. Antiproliferative activity was evaluated using xCELLigence RTCA instrument on two tumor cell lines; HT-29 (human colon adenocarcinoma) and HeLa (human cervix carcinoma). DNA damage inhibition was evaluated using photolyzing 46966 plasmid. Also, total phenolic and total flavonoid contents were determined using a spectrophotometric method. Total phenolic (343 ± 0.05 µg/mg) and flavonoid (220.5 ± 0.04 µg/mg) were indicated as gallic acid and quercetin equivalents respectively. The extract exhibited significant IC50 values in lipid peroxidation (IC50= 181.18 ± 0.65 µg/mL) and DPPH radical scavenging assays (IC50= 14.92 ± 0.032 µg/mL). The extract also partially protected 46966 plasmid DNA from free radical-mediated oxidative stress in a DNA damage inhibition assay and showed concentration-dependent antiproliferative effects. *n*-butanol extract of *L. bonduelli* is a rich source of natural antioxidants and anticancer agents. **Keywords:** *Limonium bonduelli*; Antioxidant activities; Antiproliferative activity; DNA protection.

INTRODUCTION

In recent years, there has been a great deal of attention toward the field of free radicals in biology is producing a medical revolution that promises a new age of health and disease management [1]. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species are generated by the human body by various endogenous systems, exposure to different physiochemical conditions, or pathological states, and have been implicated in the pathogenesis of many diseases [2]. Excessive production of ROS can lead to oxidative stress triggering damage in cell structures, including lipids, proteins and DNA. This damage may cause many disorders such as cancer [3]. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. Synthetic antioxidants are recently reported to be dangerous to human health. Thus the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years. In addition to endogenous antioxidant defense systems, consumption of dietary and plant-derived antioxidants appear to be a suitable alternative [4].

Plants with a long history of use in ethno- medicine can be a rich source of substances, such as polyphenols for the treatment of various chronic or infectious diseases [5].

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In the last decade, there has been much interest in the potential health benefits of dietary plant polyphenols as antioxidant. Epidemiological studies and associated meta-analyses strongly suggest that long term consumption of diets rich in plant polyphenols offers protection against the development of cancers, cardiovascular diseases, diabetes, osteoporosis, neurodegenerative diseases [6] and skin disorders [3].

Limonium species (Plumbaginaceae) have caught up the interest of researchers due to flavonoids and other phenolic components. Numerous studies on different parts of this plant demonstrate various biological activities such as antioxidant potential, anticancer [7], anti-inflammatory [8], antimicrobial 9 and hepatoprotective activities [10, 11].

Our phytochemical analysis of ethyl acetate extract from aerial parts of *L. bonduelli* revealed the presence of important secondary metabolites, flavonoids (eriodictyol, luteolin, apigenin) and 4-hydroxy-3-methoxy benzoic acid. The ethyl acetate extract of *L. bonduelli* and pure flavonoids, eriodictyol and luteolin showed strong antioxidant properties [12].

However, our literature survey revealed no published reports on the anticancer potential of *L. bonduelli* an endemic Saharan species. Thus, the present investigation aimed to explore the anticancer efficacy of *n*-butanol extract of aerial parts from *L. bonduelli* (Lestib.) Sauv. Et Vindt on two tumor cell lines; HT-29 (human colon adenocarcinoma) and HeLa (human cervix carcinoma) as well as its antioxidant and inhibition of oxidative DNA damage activities.

MATERIAL AND METHODS

Material Chemicals

Ascorbic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), $FeSO_4$, folin-ciocalteu reagent, gallic acid, H_2O_2, sodium carbonate (Na_2CO_3), thiobarbituric acid (TBA), trichloroacetic acid(TCA) and quercetin, were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum and Penicillin-Streptomycin were purchased from Sigma (Germany). All other chemicals and solvents (analytical grade) were purchased from Fluka (French).

Plant material and extraction

Aerial parts of *L. bonduelli* (Lestib.) Sauv. Et Vindt (Plumbaginaceae) were collected on April 2011 at Mogheul near Bechar in the South West of Algeria. The voucher specimen was identified by Prof. Mohamed Kaabeche from university of Setif and was deposited at the Research Unity VARENBIOMOL under the reference: LB/236/04-11.

Air-dried and powdered aerial parts (leaves and flowers, 1500 g) of *L. bonduelli* were macerated at room temperature with MeOH–H_2O (70:30, v/v) for 24 h, three times. After filtration, the combined filtrates were concentrated in vacuum (up to 35°C), the remaining solution (400 mL) was dissolved in distilled H_2O (600 mL) under magnetic stirring and maintained at 4°C for one night to precipitate a maximum of chlorophylls. After filtration, the resulting solution was extracted successively with $CHCl_3$, EtOAc and *n*-butanol. The organic phases were dried with Na_2SO_4, filtered using common filter paper and concentrated in vacuum (35°C) to obtain the following extracts: $CHCl_3$ (1.5 g), EtOAc (13 g) and *n*-BuOH (42 g) 12.

Determination of Total Phenolic Content

The total phenolic content was determined using the Folin-Ciocalteu colorimetric method described by Singleton et al. (1999) [13]. Briefly, 20 µL of sample were diluted with 1580 µL of distilled water and then mixed with 100 µL of 2 N Folin-Ciocalteu reagents. The contents were vortexed for 10 sec and then left to stand at room temperature for 6 min before the reaction was stopped by adding 300 µL 20% (w/v) sodium carbonate solution. The mixture was then incubated at 20 °C for 2 hours. The solution absorbance was measured at

765 nm. Gallic acid concentrations ranging from 50–500 µg/mL were prepared and the calibration curve was obtained using a linear fit (R^2 = 0.991). The samples were analyzed in triplicate. The concentration of total phenolic compounds was determined as µg of gallic acid equivalents (GAE) per 1 mg of extract.

Determination of Total Flavonoid Content

The total flavonoids were estimated by the aluminum chloride method described by Wang et al. (2008) [14]. Briefly, 500 µL of the sample, 500 µL of $AlCl_3$ (2%) methanol solution was added. The absorbance measured at 420 nm after 1 h incubation at room temperature. Quercetin concentrations ranging from 0 to 1200 µg/mL were prepared and the standard calibration curve obtained using a linear fit (R^2 = 0.990). The samples were analyzed in triplicate. Flavonoid content was calculated as µg of quercetin equivalents (QE) per 1 mg of extract.

Free Radical DPPH Scavenging Assay

The DPPH assay was carried out according to the procedure of Braca et al. (2001) [15], with minor modifications. In this study, different volumes (1, 2.5, 5, 10, 20, 30, 50 and 75 µg/mL) of the extract were mixed with a methanolic solution of DPPH radical (0.004%, 3mL). The mixture was kept in the dark. 30 min later, the absorbance was measured at 517 nm. The free radical scavenging activity is expressed as the inhibition percentage of free radicals by the sample and calculated using following the formula:

% of DPPH radical scavenging effect = $[(A_{control} - A_{sample})/A_{control}] \times 100$

Where A control is the absorbance of control and A sample is the absorbance of sample at 517 nm.

The IC_{50} (the concentration of antioxidant which eliminate 50% of DPPH radicals) was defined for the extract and control standard antioxidant (vitamin C). The study was carried out in triplicate at each concentration (n = 3).

Assays of lipid peroxidation using vitellus

The inhibition of lipid peroxidation was determined by quantification of MDA decomposed from the lipid peroxide, which is based on the egg *vitellus* reacting with thiobarbituric acid. For the in vitro study, the fresh *vitellus* was dissected and homogenized in ice cold PSB (20 mM, pH 7.4) to produce a 10% homogenate (v/v). The homogenate was centrifuged at 4000 rpm for 20 min to remove precipitation. 1mL aliquots of the supernatant were incubated with the extract in the presence of $FeSO_4$ (0.07 M) at 37 ºC for 1 h. The reaction was stopped by the addition of 1 mL trichloroacetic acid (TCA, 20%, w/v) and 1.5 mL thiobarbituric acid (TBA, 1%, w/v) in succession, and the solution was then heated at 100 ºC for 15 min. After centrifugation at 4000 rpm for 20 min to remove precipitated protein, the color of the complex was detected at 532 nm. The control group was run in parallel without sample under similar conditions, except that 1 mL trichloroacetic acid (TCA, 20%, w/v) was added before incubation and all measurements were done in triplicate. The lipid peroxidation scavenging activity (K %) was calculated by the following equation:

$K\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$

Where A control is the absorbance of control and A sample is the absorbance of sample [16].

DNA damage inhibition efficiency

The potential of *n*-butanol extract of *L. bonduelli* to inhibit DNA damage was tested by photolyzing 46966 plasmid DNA (extracted from Escherichia coli) via UV radiation in the presence of H_2O_2 and performing agarose gel electrophoresis with the irradiated DNA17. Into two polyethylene's micro centrifuge tubes, 1µL aliquots of 46966 plasmid (0.2 µg/mL) were added, followed by 50 µg of n-butanol extract in one of the two tubes without the other which the irradiated control. Then 4 µL of 3% H_2O_2 was added into the two tubes, which were then placed directly on the surface of a UV transilluminator (300 nm) during 10 min at

room temperature. In another tube, 1 µL aliquot of 46966 plasmid DNA was placed and served as the non-irradiated control. All the samples were run on 1% agarose gel and then photographed using a Lourmat gel imaging system (Vilber) [17].

Evaluation of the anticancer activity using xCELLigence system

Cell culture and preparation of Cell Suspension

HT-29 (human colon adenocarcinoma) and HeLa (human cervix carcinoma) cells were grown in Dulbecco's modified eagle's medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and 2% Penicillin-Streptomycin (Sigma, Germany) at 37°C in a 5% CO_2 humidified atmosphere. Cells lines in the culture flask were detached from bottom of flask by 10 mL Trypsin-EDTA solution. After detachment, 10 mL of medium was added into the flask and mixed thoroughly. This suspension was transferred to Falcon tubes and centrifuged at 600 rpm for 5 min.

After removing the supernatant, 5 mL of medium was added to Falcon tube and mixed carefully. Cell concentration of this cell suspension was measured by CEDEX HiRes Cell Counter which uses Trypan Blue [18].

Preparation of E-Plate 96 and treatment

50 µL of medium was added into each well of E-Plate 96. The plate was incubated in the hood, then in the incubator for 15 min in each time. After this period, the E-Plate 96 was inserted to the RTCA-SP station and a background measurement were performed. Then 100 µL of the cell suspension ($2.5x10^4$ cells/100 µL) was added into the wells, except in the last three wells were only 100 µL of medium was added to check if there would be an increase from culture medium. The plate was left in the hood for 30 min, and then inserted into the xCELLigence station in the incubator. A measurement was performed for 80 min. Extract was dissolved in DMSO to a final concentration of 20 mg/mL. 25 µL of this solution was mixed with 475 µL of medium. The extract solutions (50, 20 and 10 µL equivalent to 250, 100 and 50 µg/mL concentrations, respectively) were added into the wells and the final volumes were completed to 200 µL with medium. No extract solution was added into control and medium wells. Then the plate was inserted to xCELLigence station in the incubator and a measurement for 48h or 72h was started [18].

Statistical analysis

All data are expressed as the mean ± SD of triplicate measurements. The statistically significant differences between mean values at the level of significance ($P < 0.05$) were evaluated by the paired t test in SPSS (version 19.0).

RESULTS AND DISCUSSION

Total phenolic compounds

Phenols are very important secondary metabolite plant constituents because of their scavenging capacity on free radicals due to their hydroxyl groups [19, 20]. Therefore, it is worthwhile to determine the total amount of phenolic content in the plant chosen for the study. The total phenolic content was determined by Folin–Ciocalteu reagent in terms of gallic acid equivalents used the regression equation of the calibration curve obtained from gallic acid ($y = 0.001x$, $R^2 = 0.991$). The results showed that the *n*-butanol extract of *L. bonduelli* possesses high levels of total phenolic contents (343 ± 0.05 µg/mg).

Total flavonoids content

Flavonoids have been identified as active constituents of medicinal plants. Due to their beneficial effects on human health, this class of compounds has received a great deal of attention, not only from the pharmaceutical industry, but also from other areas of applied

sciences, including the food, brewing, and cosmetics industries [21]. The concentration of flavonoids in *n*-butanol extract of *L. bonduelli* was determined using the spectrophotometric method with aluminum chloride ($AlCl_3$). The content of flavonoids was expressed in terms of quercetin equivalents used the regression equation of the calibration curve obtained from quercetin ($y = 0.034x + 0.015$, $R^2 = 0.990$). Whereby the flavonoids content was (220.5 ± 0.04 µg/mg).

The results obtained indicated that *n*-butanol extract of of *L. bonduelli* is a rich source of antioxidants, including phenols and flavonoids. In general, the higher phenolic contents resulted in higher antioxidant activity [20].

DPPH radical scavenging activity

The presence of phenolic compounds like flavonoids in the extract is probable to be responsible for the free radical scavenging activity. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of free radicals [22]. In the present study, the free radical scavenging activity of *n*-butanol extract of *L. bonduelli* and vitamin C were evaluated through their ability to quench 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. Figure 1 illustrates a significant decrease of DPPH radical due to the scavenging ability of tested samples which correlates with a dose effect dependence. Compared to vitamin C (IC_{50}=5±0.1 µg/mL), *n*-butanol extract showed a considerable scavenging activity (IC_{50}=14.92±0.032 µg/mL) and the highest percentage of the inhibition was 96% similar to vitamin C (Figure 1) in the same concentration (25 µg/mL). The presence of phenolic compounds (containing phenolic hydroxyls) are mainly found in this extract and could be attributable to the observed high antiradical properties. In this context, several authors reported that most antioxidant activities from plant sources are derived from phenolic compounds. Structurally, phenolics comprise an aromatic ring, bearing one or more hydroxyl substituent. The antioxidant activity of this kind of molecule is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations [3].

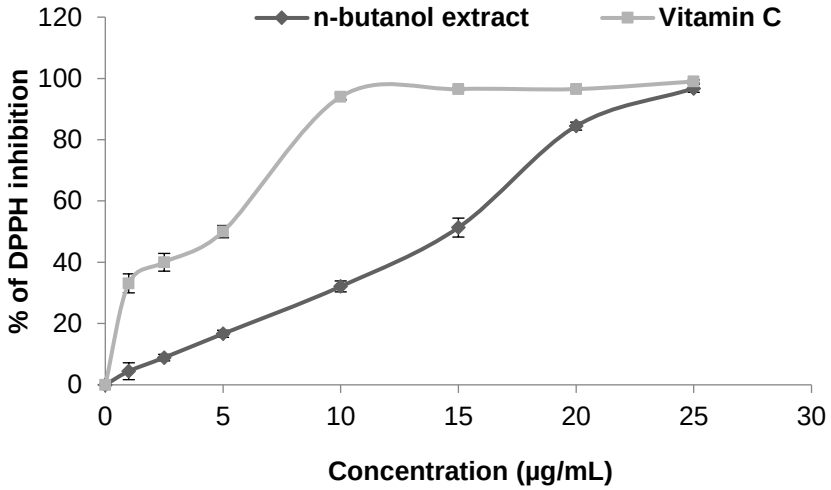

Figure 1. DPPH Scavenging activities of *n*-butanol extract of *L. bonduelli* and vitamin C. Values are mean ± SD of three samples analyzed individually in triplicate.

Inhibition of lipid peroxidation

In biological systems, lipid peroxidation generates a number of degradation products, such as MDA, and it was considered to be an important cause of cell membrane destruction and cell damage [23]. This study measured the potential of *n*-butanol extract of *L. bonduelli*

to inhibit lipid peroxidation in egg *vitellus* homogenate induced by the $FeSO_4$ system. Clearly, the *n*-butanol extract and Vitamin C (standard) inhibited lipid peroxidation in a dose dependent manner as shown in Figure 2. The Vitamin C produced greater inhibition (IC_{50} = 20 ± 0.40µg/mL) as compared to the extract (IC_{50} = 181.18 ± 0.65 µg/mL). *n*-butanol extract of *L. bonduelli* demonstrated moderate anti-lipid peroxidative effects, which may be useful in preventing the progress of various oxidative stress related diseases such as inflammation and cancer. Phenolic compounds are majorly responsible for the antioxidant activity of plant materials [24]. Flavonoids are strong metal chelators that inhibit lipid peroxidation [25].

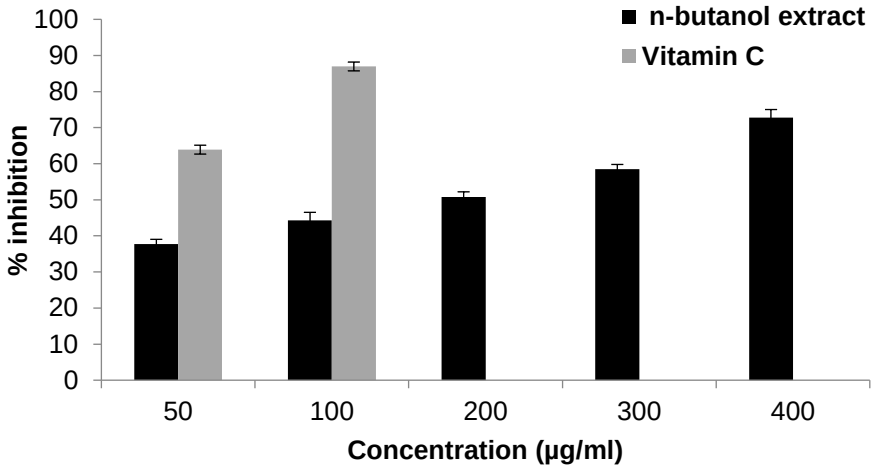

Figure 2. Inhibition by *n*-butanol extract of *L. bonduelli* and vitamin C of $FeSO_4$ induced lipid peroxidation of egg *vitellus*. Values are expressed in terms of mean ± SD for three observations.

DNA damage inhibition efficiency

In case of plasmid, damage of DNA results in a cleavage of one of the phosphodiester chains of the supercoiled DNA and produces a relaxed open circular form. Further cleavage near the first breakage results in linear double-stranded DNA molecules. The formation of circular form of DNA is indicative of single-strand breaks and the formation of linear form of DNA is indicative of double-strand breaks [26, 27]. In this work we evaluate the oxidative DNA damage protective activity of *n*-butanol extract of *L. bonduelli* on DNA cleavage induced by H_2O_2 UV-photholysis using *in vitro* method of Russo et al. (2001) [17]. UV-photolysis of H_2O_2 generates $^{\bullet}OH$ radicals, which cause colossal oxidative damage. $^{\bullet}OH$ bound to DNA leads to strand breakage, deoxysugar fragmentation, and base modification. As shown in lane 2 of Figure 3 UV-photolysis of H_2O_2 of 46966 plasmid DNA resulted in the cleavage of supercoiled to give open circular (OC DNA) and linear forms of plasmid DNA (LIN DNA), indicating that $^{\bullet}OH$ generated from UV-mediated decomposition of H_2O_2 produced both single-strand and double-strand DNA breaks. Addition of 50 µg/mL of *n*-butanol extract of *L. bonduelli* induced the significant reduction in the formation of open circular and linear forms and increased supercoiled or native form of plasmid DNA. The DNA cleavage analysis demonstrated the strong antioxidant properties of *L. bonduelli*. In fact, this extract suppressed the formation of linear DNA, generated by exposure of plasmid DNA to $^{\bullet}OH$ radical generated by H_2O_2 UV-photolysis, and induced a partial recovery of super coiled DNA. The extract showed high phenolic content which justified the antioxidant and DNA damage inhibition properties of the plant. This result agreed with the results of many other studies suggested that phenolic compounds can protect the DNA from the oxidative damage [28-31]. The DNA damage inhibition potential of the extract could be used in cancer prevention.

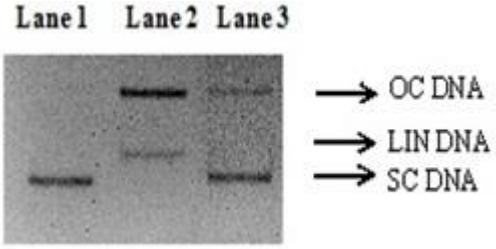

Figure 3. Effect of extract (50µg) on the protection of supercoiled DNA (46966) against oxidative damage caused by UV- photolysed H_2O (3%). Lane 1: untreated non-irradiated DNA (control); Lane 2: UV-irradiated DNA; Lane 3: UV-irradiated, n-butanol extract treated (Lb); SC DNA: supercoiled DNA; OC DNA: open circular DNA; LIN DNA: linear DNA.

Anticancer activity

There have been many reports of plant extracts and different types of phytochemicals especially phenolic compounds as secondary metabolites from plants, which were shown to have a very effective anticancer activity [32-34]. However, anticancer investigations on *L. bonduelli* have not been studied so far. The anticancer activity of *n*-butanol extract of the aerial parts (leaves and flowers) of *L. bonduelli* against HeLa (human cervix carcinoma) and HT-29 (human colon adenocarcinoma) cells was tested using xCELLigence RTCA instrument. As shown in Figure 4 and 5, the extract exhibited different profiles on different concentrations. It has the most activity with the concentration of 250 µg/mL and it was about to reach to medium after 5 h post-treatment, while other concentrations (100 and 50 µg/mL) showed less activity in a dose-dependent manner.

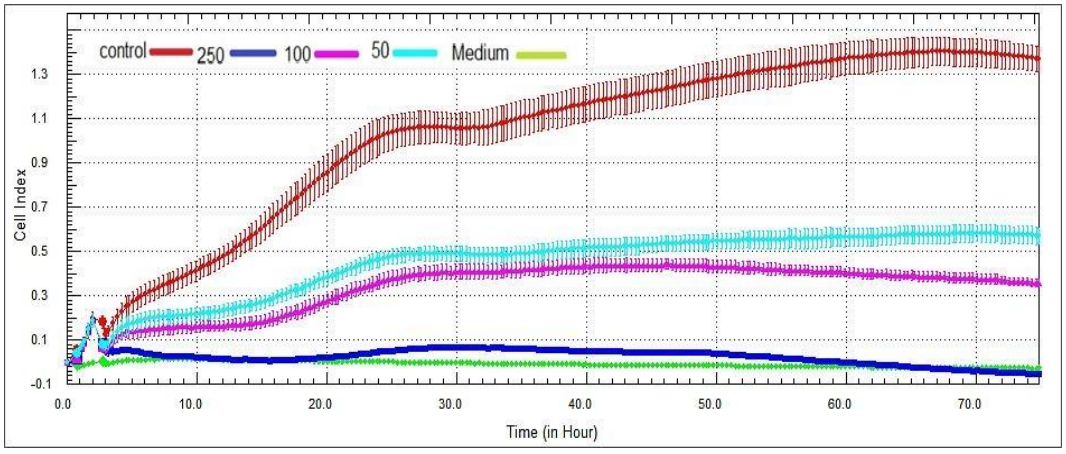

Figure 4. Anticancer activity of the *n*-butanol extract of *L. bonduelli* against HeLa cell line. Each substance was tested twice in triplicates against cell lines using xCELLigence RTCA instrument. As seen in the diagram, several extract concentrations were applied to the cells and each color represents a different concentration.

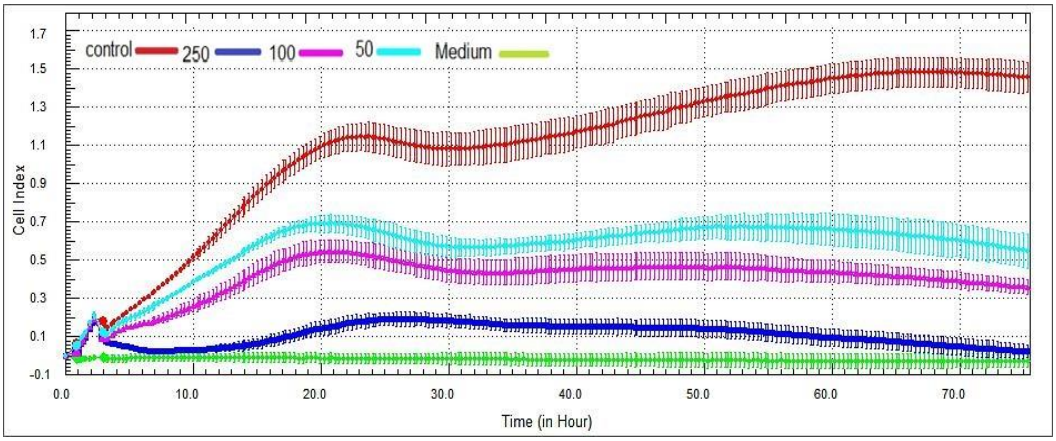

Figure 5. Anticancer activity of the *n*-butanol extract of *L. bonduelli* against HT-29 cell line. Each substance was tested twice in triplicates against cell lines using xCELLigence RTCA instrument. As seen in the diagram, several extract concentrations were applied to the cells and each color represents a different concentration.

The profiles also showed differences at different time points. Low concentrations showed better activity against HeLa cells at 15th h (Figure 4) and against HT-29 cells at 30th h (Figure 5) after post-treatment. The results clearly show that *n*-butanol extract of *L.bonduelli* significantly inhibit the proliferation of cancer cells in a concentration-dependent manner during incubation for 72 h. Moreover, the highest concentration of *n*-butanol extract of *L.bonduelli* (250 µg/ml) shows the highest inhibition in proliferation in all cell lines, where the inhibition was recorded as 92.6% and 98.9%, for HT-29 and HeLa cells, respectively. The antioxidant, antiproliferative and DNA protective abilities of the plant extract render them suitable to be considered as a source for the development of anti-cancer drugs. The antioxidant properties of the extract might prevent cancer progression; while the DNA protection property might hold good in inhibiting secondary mutations in progressive tumor tissues [35].

CONCLUSION

These experimental results provide the basis for the development of promising natural anticancer agents possessing antioxidant activity and supporting the potential use of *n*-butanol extract of *L. bonduelli* in the pharmaceutical and manufacturing industries. Further investigations are required to understand the possible mechanism(s) of action of the extract on various cancer cells and isolation of active phytochemicals. *In vivo* studies are needed to confirm the pharmacological efficacy and safety of *L. bonduelli* extract.

Conflicts of Interest

The authors declare no conflict of interest.

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