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# In Vitro Assessment of Anti-Melanoma and In Vivo, In Vitro Antioxidant Activity of Ethanol Extract of Boscia Seneglensis Leaf.

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**Abstract:** This study investigates the effectiveness of Boscia plant leaf (BSL) extract in treating melanoma skin cancer. The leaf of the Boscia plant was extracted using ethanol, and various concentrations of BSL were tested. The P values of five concentrations were obtained as $P<0.0001$, $P<0.002$, $P<0.002$, $P<0.001$, and $P<0.0033$, indicating significant cytotoxic effects on the tested samples. The research suggests that further soaking of the sample extract during an incubation period exceeding 48 hours could yield more substantial results. Additionally, the impact of the culture medium varied during the growth time. Furthermore, the study explored the effect of antioxidants on the liver, showing promising results that warrant further investigation.

**Keywords:** Boscia Seneglensis, Antioxidants, Skin Cancer, LDH, Trypan blue

## 1. Introduction:

Assessing medicinal plants' antioxidant and anti-cancer potential primarily relies on in vitro investigations[1]. Finding efficient anti-melanoma treatments is a continuous quest for skin cancer, especially melanoma.[2], is a severe health concern. Since oxidative stress is strongly linked to the development of skin cancer[3]Antioxidants have demonstrated promise in reducing their effects. Medicinal plants have been of particular interest due to their rich phytochemical composition,

which includes compounds with potential anti-cancer and antioxidant activities[4]. This research aims to investigate the in vitro anti-melanoma and antioxidant properties of the ethanol extract of Boscia seneglensis leaf.[5], shedding light on its potential as a natural anti-cancer and antioxidant agent for skin health[6]."

To research the characteristics and actions of particular cell types[7], cell lines are an essential tool in biomedical research. In anti-cancer and antioxidant research, using cell lines allows for investigating the effects of medicinal plant extracts on cancerous and normal cells.[8]. Choosing appropriate cell lines is crucial for obtaining relevant and reliable data regarding natural substances.'[9] Potential anti-melanoma and antioxidant properties. In this study, selecting specific cell lines will play a key role in providing insights into the efficacy of the ethanol extract of Boscia seneglensis leaf as a potential anti-cancer and antioxidant agent for skin health.[10].

## 2.Materials & methods:

All of the chemicals and reagents used in the study were of the molecular biology and analytical grades[11], and they were all acquired from Everon Life Sciences -New Delhi. Boscia senegalensis leaf were purchased from ARC-Sudan. The standard medication, fluorouracil 5%, was obtained from Unique Health Pharmacy in Delhi. Trypan Blue was purchased from Quesst International-Bengaluru. Cell line (Melanoma RPMI7951) Ref No: 149/2023-24 was obtained from the National Centre for Cell Science, Pune-India.

### 2.1In vitro anticancer studies

#### *a.Trypan blue dye exclusion assay*

It is the most utilized test for cell viability. In this assay, the cells are washed with HBSS (Hank's Buffered Salt Solution)[12] and centrifuged for 10 - 15 min at 10,000 rpm. The procedure is repeated thrice. The cells are suspended in a known quantity of HBSS, and the cell count is adjusted to 2 x 106 cells /ml. The cell suspension is distributed into Eppendorf tubes (0.1ml containing 2 lakh cells). The cells are incubated for three hours at 37 °C after being exposed to drug dilutions. Trypan blue (0.4%) is mixed with equal-quality drug-treated cells and left for one minute in the dye exclusion test after three hours. After that, it is loaded into a hemocytometer and viable and non-viable counts are recorded in two minutes. After that, it is loaded into a hemocytometer and viable

and non-viable counts are recorded in two minutes. After that, it is loaded into a hemocytometer and viable and non-viable counts are recorded in two minutes[13]. After that, it is loaded into a hemocytometer and viable and non-viable counts are recorded in two minutes. After that, it is loaded into a hemocytometer and viable and non-viable counts are recorded in two minutes[14]. Colour is absorbed by dead cells but not by viable cells[15]. Still, living cells produce and absorb colour if they are preserved longer. To compute the growth inhibition percentage, utilize the following formula[16]:

$$Growth\ inhibition(\%) = 100 - \frac{(Total\ Cells - Dead\ Cell)}{Total\ Cells} \times 100$$

***b.LDH (Lactic Dehydrogenase assay)***

Using spectrophotometry, the amount of lactic dehydrogenase in the culture medium and cellular lysates is measured at 340 nm by examining the reduction of NADH during the pyruvate lactate transformation.[17]. The cells are further broken down using sonication, then they are lysed in 50 mM Tris-HCl buffer, pH 7.4 + 20 mM EDTA + 0.5% Sodium Dodecyl Sulfate (SDS), and centrifuged for 15 minutes at 13,000 X g. The assay mixture (1ml final volume) for the enzymatic analysis consists of 33 µl of sample in 48 mM PBS, pH 7.5 + 1mM pyruvate, and 0.2 mM NADH. The percentage of total amount, which is the sum of the enzymatic activity in the culture medium and the cellular lysate, is used to calculate the percentage of LDH released.[18].

**2.2 In vitro Antioxidant studies**

***a. DPPH assay***

Using 1,1-diphenyl-2-picrylhydrazyl (DPPH), the DPPH radical scavenging assay was conducted by the protocol outlined by Brand-Williams et al.[19]. The plant extracts under study were used to produce analytical-grade methanol at five distinct concentrations (0.2, 0.4, 0.6, 0.8, 0.8, and 1 mg/ml). Five were prepared with methanol (analytical grade). L-ascorbic acid, a common antioxidant, was also produced in the same amounts. One millilitre

of every examined extract was moved into a sterile test tube, and then 0.5 millilitre of 0.3-millimetre DPPH in methanol were added. After giving the mixture a good shake, it was left to lie at room temperature for fifteen minutes in the dark. As a baseline, blank solutions comprised 2.5 ml of the investigated extract solutions and 1 ml of methanol were utilized. L-ascorbic acid was

utilized as the positive control at concentrations equivalent to those of the extracts under study, while 2.5 ml of DPPH solution and 1 ml of methanol served as the negative controls[20]. A spectrophotometer was used to measure the absorbance values at 517 nm following a dark incubation period. The experiments were performed in triplicate. The activity of DPPH radical-scavenging was estimated using the formula given by Brand-Williams et al[21].

***b. TBA assay***

By using the method Ottolenghi (1959), it is as follows: This procedure was performed with a final sample concentration of 0.02% w/v. One milliliter of the BSL extract sample solution was mixed with 20% trichloroacetic acid and two milliliter of 0.67% thiobarbituric acid. After cooling for 20 minutes at 3000 rpm, the mixture was centrifuged after being immersed in boiling water for 10 minutes. After reaching its peak, the absorbance activity of the supernatant was measured at 552 nm.

***c.$H_2O_2$ assay***

Humans are exposed to 0.28 mg/kg of H2O2 in the environment on a daily basis, mostly through leaves. Calculating the hydrogen peroxide scavenging capacity of plant extracts can be done using the Ruch et al. (1989) method[22]. crops. The human body can absorb hydrogen peroxide through the eyes, skin, or inhalation of vapour or mist. Hydroxyl radicals (OH), which can initiate lipid peroxidation and harm DNA within the body, can be produced when H2O2 breaks down quickly into oxygen and water. Plant extracts' capacity to scavenge hydrogen peroxide can be approximated using the Ruch et al. (1989) technique. 50 mM of pH 7.4 phosphate buffer creates a 40 mM hydrogen peroxide solution. Using a spectrophotometer, the absorption at 230 nm yields the hydrogen peroxide concentration[23]. Hydrogen peroxide is mixed with extract (20–60 lg/mL) in distilled water, and after 10 minutes, absorbance at 230 nm is measured compared to a blank solution that contains phosphate buffer but no hydrogen peroxide. The following formula can be used to determine the percentage of hydrogen peroxide scavenging[24].

% scavenged ($H_2O_2$) =[ ($A_t$-$A_t$)] /$A_i$ x 100

**2.3 In vivo antioxidant studies**

***Preparation of tissue Homogenate***

Following the collection of blood, an overdose of 100 mg/kg (IP) pentobarbitone was used to sacrifice every animal. Isolate the liver, wash it with ice-cold saline, and homogenize it with distilled water.[25]. The supernatant solution was collected in a second centrifuge tube and stored at -20˚C for use in antioxidant study measurements, such as total protein, catalase, and superoxide dismutase.[26].

**a. Superoxide dismutase (SOD)**

The Kakar et al. method was used to estimate SOD activity. Add 186 µmol, 1.2 ml of phosphate buffer (0.052 mmol; pH 7.0), and 0.3 ml of liver tissue homogenate to 0.1 ml of phenazine methosulphate[27]. The enzyme reaction was stopped after one minute and the addition of 0.2 ml of NADH (780 µmol) and 1 ml of glacial acetic acid. The chromogen formation amount was measured at 560 nm. Results are expressed in units/mg protein[28].

**Calculation,**

$$\frac{\mathrm{c} \times \text{total volume} \times 1000}{50 \times \text{sample volume} \times \text{mg of protein}}$$

$$C = \frac{\mathrm{x} + \mathrm{y} + \mathrm{z}}{3}$$

Total volume – 3 mL Abs. at 0 min – x

Sample volume - 0.1mL Abs. at 1st min –y

Abs. at 2nd min –z

$$\frac{\mathrm{c} \times 3 \times 1000}{\text{mg of protein} \times 0.1 \times 50}$$

**b. Catalase Estimation**

100ul of supernatant was added to 1.9 ml phosphate buffer (Ph7) and zero main absorbance was measured at 240 nm. Add one milliliter of H2O2 to the solution above. Using phosphate buffer as a blank, measure the change in absorbance at 240 nm for two minutes[29]. One IU of catalase utilized is that amount which catalyzes the decomposition of 1mM hydrogen peroxide per min at 37°C and

expressed in terms of Units/mg of proteins. The consumption of H2O2 was monitored spectrophotometrically at 240 nm for 1min. The enzymatic activity is computed using the formula below[30]:

K = 2.303/T x log (A1/A2)

Where; K: Rate of reaction

T: Time interval (minutes)

A1: Absorbance at time zero

A2: Absorbance at 60 seconds interval

Calculation,

$$T \times \frac{diluation\ factor \times 100}{\text{abs. at 0 sec} \times \text{mg of protein}}$$

Dilation factors:100 $\underline{T \times 100 \times 100}$

$abs\ at\ sec. \times mg\ of\ protein$

**Results & discussion**

Table 1, Cytotoxicity of $10 \mu g/ml$ of ethanol extract of BSL in melanoma RPMI7951.

| S.NO | Total cell counted | Live concentration | Live concentration | Dead concentration | Viability (%) |
|---|---|---|---|---|---|
| 0 | 254007 | 4329 | 630 | 217007 | 14.55 |
| 1 | 181007 | 3085 | 237 | 167007 | 7.68 |
| 2 | 153007 | 2603 | 138 | 145007 | 5.30 |
| 3 | 52800 | 9 | 3 | 35200 | 33.33 |
| 4 | 254007 | 4329 | 630 | 217007 | 14.55 |
| 5 | 181007 | 3085 | 237 | 167007 | 7.68 |

P< 0.0001 indicates significant differences in the treatment group.

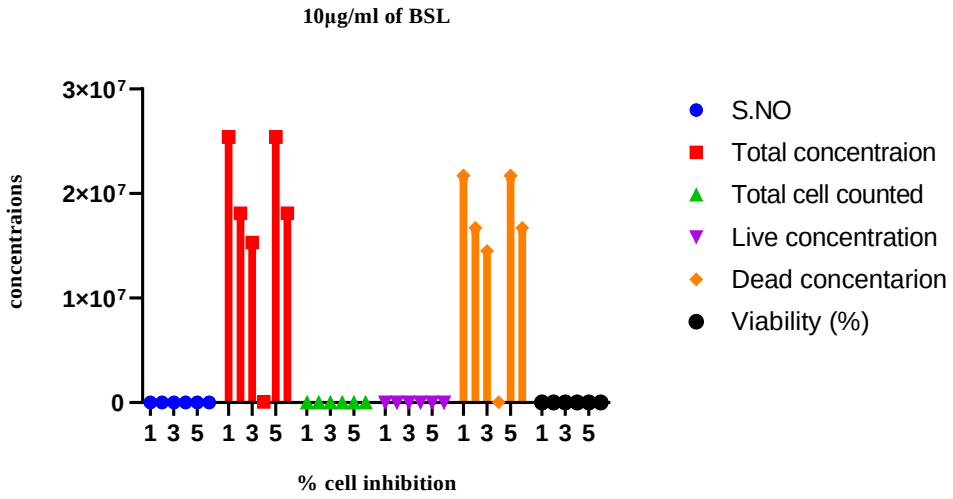


Figure 1, Growth inhibition of BSL against RPMI7951, on $10\mu g/ml$

Table 2, Cytotoxicity of $20\mu g/ml$ of ethanol extract of BSL in melanoma RPMI7951.

| S.NO | Total cell counted | Live concentration | Live concentration | Dead concentration | Viability (%) |
|---|---|---|---|---|---|
| 0 | 1380000 | 76200 | 1300000 | 222 | 5.531915 |
| 1 | 1460000 | 358000 | 1100000 | 188 | 24.49799 |
| 2 | 1460000 | 358000 | 1100000 | 188 | 24.49799 |
| 3 | 136000 | 704000 | 129007 | 2196 | 5.181347 |
| 4 | 136000 | 704000 | 129007 | 2196 | 5.181347 |
| 5 | 151000 | 2150000 | 129007 | 2201 | 14.29128 |

P<0.002 indicates significant differences in the treatment group.

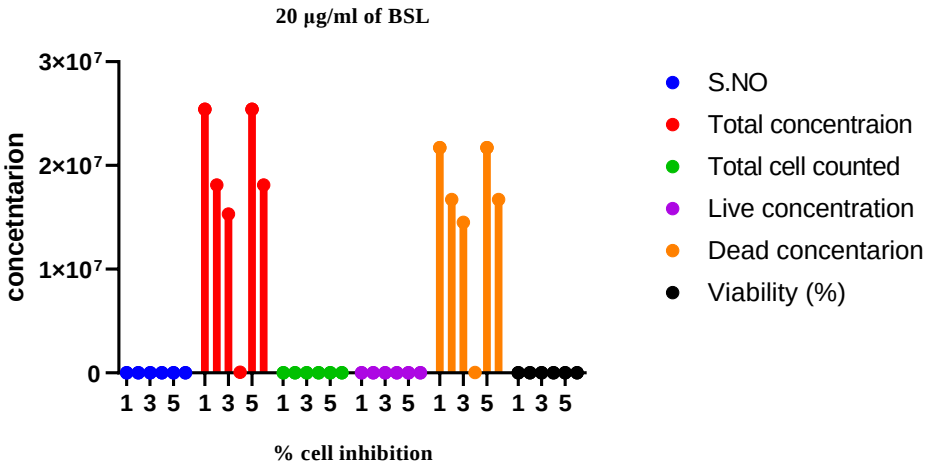


Figure 2, Growth inhibition of BSL against RPMI7951, on 20µg/ml

Table 3, Cytotoxicity of 30$\mu g/ml$ of ethanol extraxt of BSL in melanoma RPMI7951.

| S.NO | Total concentration | Total cell counted | Live concentration | Dead concentration | Viability (%) |
|---|---|---|---|---|---|
| 0 | 1640000 | 280 | 323000 | 1320000 | 19.6 |
| 1 | 369000 | 63 | 270000 | 99700 | 73.01 |
| 2 | 156007 | 2666 | 1890000 | 137007 | 12.11 |
| 3 | 473007 | 8061 | 5540000 | 417007 | 11.71 |
| 4 | 243007 | 4145 | 2990000 | 213007 | 12.27 |
| 5 | 15607 | 2666 | 18906 | 13707 | 12.11 |

P<0.002 indicates significant differences in the treatment group

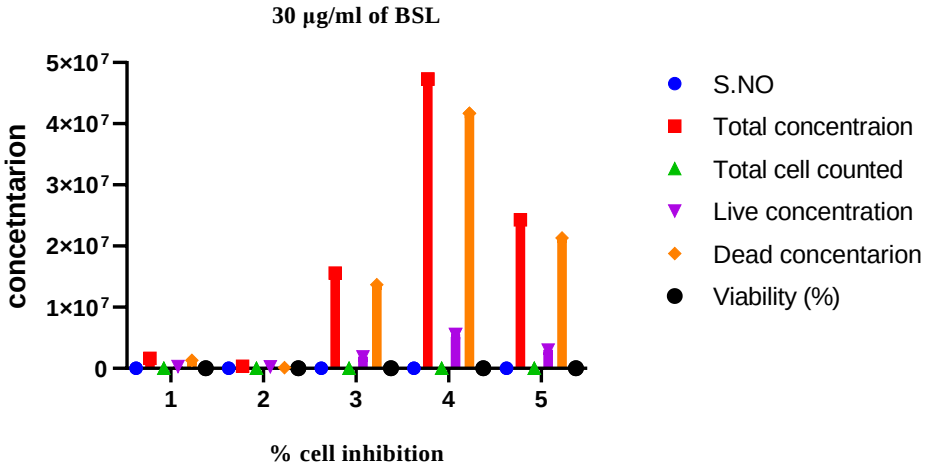


Figure 3, Growth inhibition of BSL against RPMI7951, on 30µg/ml.

Table 4, Cytotoxicity of 40 µg/ml of ethanol extract of BSL in melanoma RPMI7951.

| S.NO | Total concentration | Total cell counted | Live concentration | Dead concentration | Viability (%) |
|---|---|---|---|---|---|
| 0 | 77206 | 1317 | 32706 | 44506 | 42.36 |
| 1 | 16606 | 283 | 45705 | 12006 | 27.56 |
| 2 | 16506 | 282 | 44605 | 12106 | 26.95 |
| 3 | 12807 | 2187 | 29905 | 12507 | 2.33 |
| 4 | 11607 | 1980 | 34605 | 11307 | 2.99 |

P< 0.001 indicates significant differences in the treatment group.

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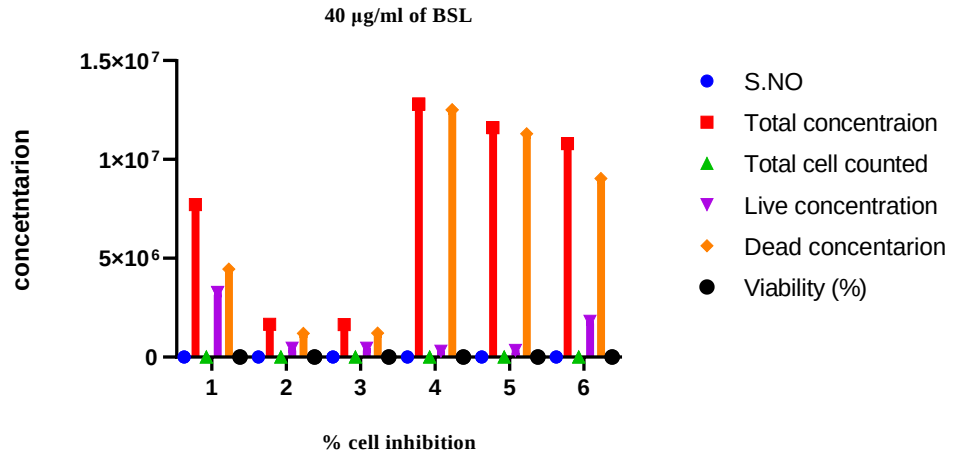



Figure 4, Growth inhibition of BSL against RPMI7951,on 40µg/ml.

Table 5, Cytotoxicity of 50 µg/ml of ethanol extract of BSL in melanoma RPMI7951

| S.NO | Total concentration | Total cell counted | Live concentration | Dead concentration | Viability (%) |
|---|---|---|---|---|---|
| 0 | 88005 | 150 | 54005 | 34005 | 61.33 |
| 1 | 88005 | 150 | 54005 | 34005 | 61.33 |
| 2 | 12207 | 2087 | 57505 | 11707 | 4.69 |
| 3 | 13107 | 2234 | 13406 | 11807 | 10.25 |
| 4 | 20207 | 3444 | 10306 | 19207 | 5.081 |
| 5 | 28007 | 4772 | 63905 | 27307 | 2.28 |

P<0.003 indicates significant differences in the treatment group.

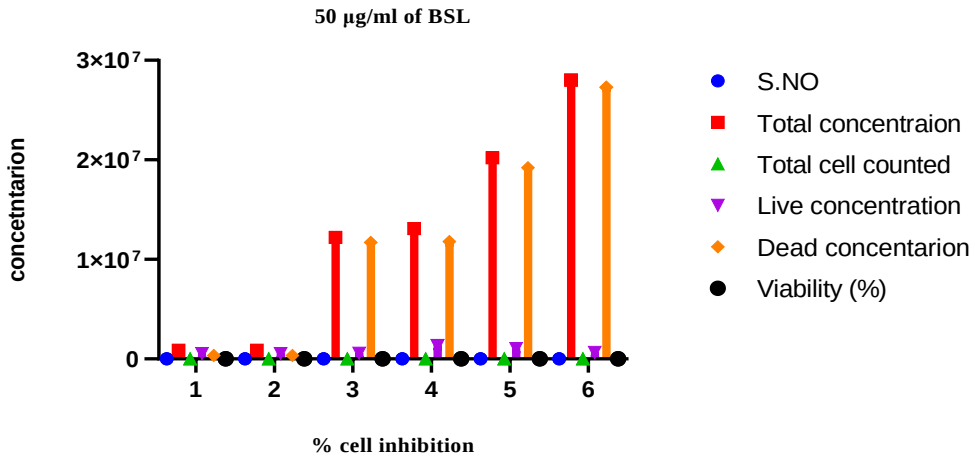



Figure 5, Growth inhibition of BSL against RPMI7951, on 50µg/ml.

***LDH***

According to data derived from the research, the effectiveness of BSL on melanoma skin cancer of several tested samples demonstrated a significant cytotoxic effect. However, the sample extract could show a more significant result if it is soaked more during incubation time, which is more than 48 hours, but the effect of the culture medium could also vary during growth time.

Table 6, Cytotoxicity activity of BSL extracts against RPMI7951cell line at different concentrations by LDH Assay.

| S.No | Conc-of BSL mg/ml | Absor Mean±SEM | % Inhibition | IC50, mg/ml |
|---|---|---|---|---|
| S-1 | 0.1 | 0.012 ±0.01 | 50.33 | - |
| S-2 | 0.2 | 0.026 ±0.02 | 24.33 | - |
| S-3 | 0.3 | 0.024 ±0.03 | 18.55 | - |
| S-4 | 0.4 | 0.029 ±0.01 | 25.30 | - |
| S-5 | 0.5 | 0.032 ±0.02 | 22.62 | 0.61 |

The percentage of cell inhibition represented in the curve as below vs. the concentration of extract on melanoma skin cancer (RPMI7951) showed a higher significance value of cell inhibition. The cell underwent different stages of morphological change during different incubation times, which the cell wall's cytotoxicity may have caused. DNA is the main mechanism for cell growth and synthesis promotion, ultimately influencing outgoing growth and cell synthesis via cell communication.

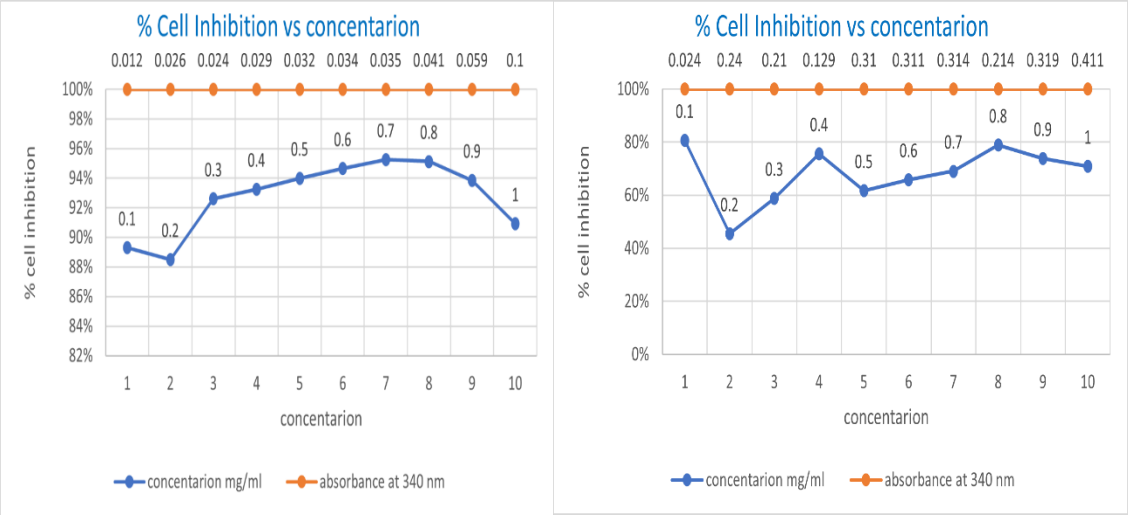
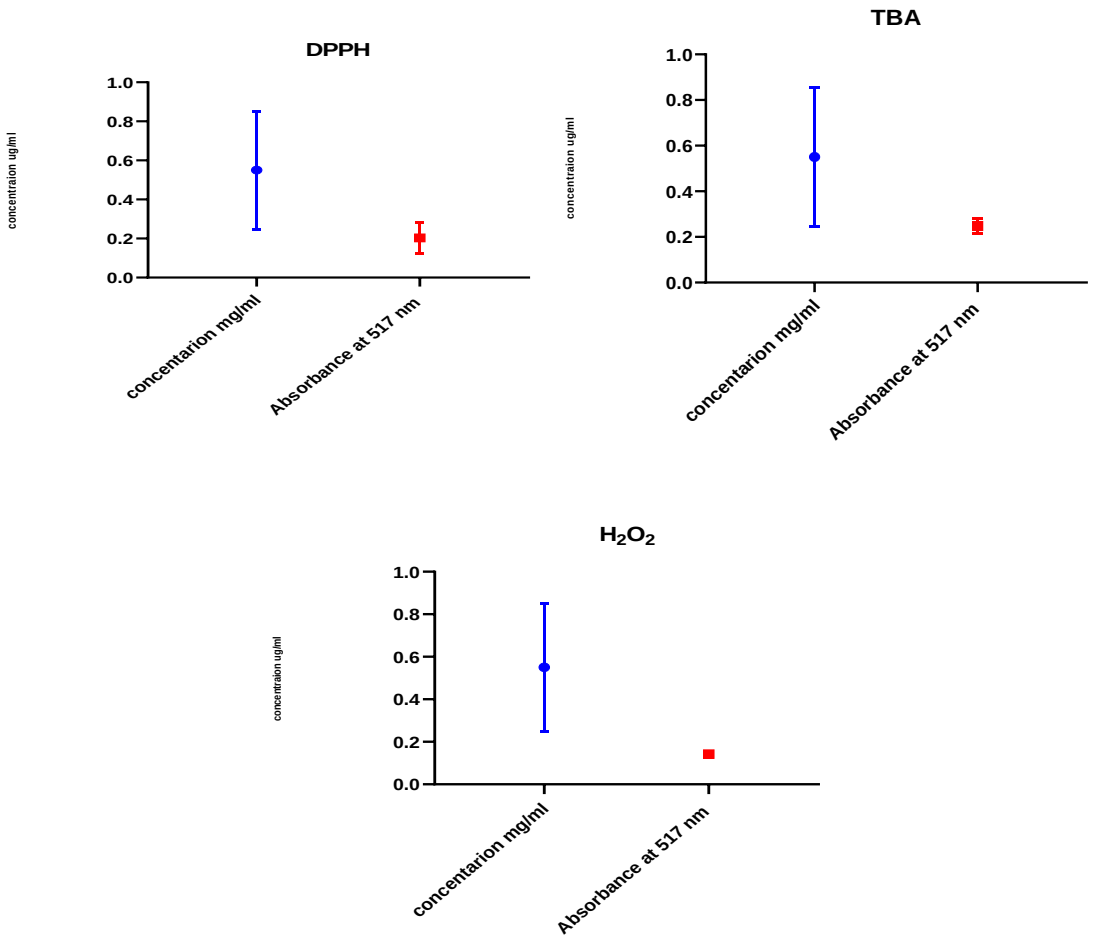



Figure 6, Growth inhibition of BSL against RPMI7951melanoma skin cancer by LDH assay.Table 7, Effect of BSL on antioxidant enzymes in liver tissue

| Experimental Group | DPPH Assay | TBA Assay | $H_2O_2$ Assay |
|---|---|---|---|
| Normal Group | 5.1±1.12 | 87.6 | 9.6±0.66 |
| DMBA-TPA | 2.38±1 | 31.6±4.8 | 13.4±3.4 |
| DMBA-TPA+Fu-5 | 3.2±5.5 | 83.8±9.4 | 8.1±2.5 |
| DMBA-TPA+BSL | 4.03±5.5 | 61.4±4.5 | 7.2±0.7 |

All values are expressed as mean. ± SD. Data were analyzed using one-way ANOVA followed by Dunnett's t-test, P value $P<0.05$, $P<0.028$, $P<0.001$.




The antioxidant content of BSL extract was significantly higher in BSL ethanol extract by the DPPH assay, which is well known for its ability to measure antioxidants in plant extracts. Compounds or minute particles known as free radicals, or ROS, have one or more unpaired electrons in their molecular or atomic orbitals. They also contain a variety of oxygen species, including hydrogen peroxide, which aids in the fight against cancer. Compared to the H2O2 assay method, the BSL extract of different samples was found in the DPPH assay method $P<0.001$. In contrast to the DPPH and H2O2 assays, the BA assay indicates that the BSL extract contains fewer antioxidants. The antioxidant activity of BSL ethanol extracts from investigated plants obtained by DPPH, TPA, and $H_2O_2$ is highest in BSL extracts. However, our research has revealed a fascinating complexity: different pathways for producing antioxidants can be found in different parts of BSL, shedding new light on their intricacies.

**Conclusion:**

Based on the results, it is evident that the plant extract of BSL demonstrates significant cytotoxicity at various concentrations, particularly at 10 ug/ml and 20 ug/ml. The incubation period potentially influences the cytotoxicity of melanoma cells. Additionally, the BSL extract displayed potent antioxidant properties in both in vivo and in vitro studies. Further research is necessary to elucidate the specific mechanism of BSL and determine the exact percentage of inhibition in melanoma cells

**Acknowledgment: Nil**

**Reference:**

1. Alhalouly T, Landry D, et al. Pagetoid Variant of Actinic Keratosis with or without Squamous Cell Carcinoma of Sun-Exposed Skin: A Lesion Simulating Extramammary Paget's Disease. Histopathology 2023;41:331–6.

2. Barreto JE, Velazquez EF, Ayala E, et al. Carcinoma Cuniculatum: A Distinctive Variant of Penile Squamous Cell Carcinoma: Report of 7 Cases. Am J Surg Pathol 2017;31:71–5.

3. Bowra GT, Duffield DP, Osborn AJ, et al. Premalignant and Neoplastic Skin Lesions Associated with Occupational Exposure to "Tarry" Byproducts during Manufacture of 4,4'-Bipyridyl. Br J Ind Med 2019;39:76–81.

4. C.A. Finlay, P.W. Hinds, A.J. Levine (2019). The P53 Proto-Oncogene Can Act as a Suppressor of Transformation. Cell, 57, 1083–1093.

5. Akanji, M.A., Olagoke, O.A.and Oloyede, O.B.. Effect of Chronic Con Sumption of Metabi-Sulphite on the Integrity of the Kidney Cellular System. Toxicology,2019-81:173-179.

6. Bendl BJ, Graham JH. New Concepts of the Origin of Squamous Cell Carcinomas of the Skin: Solar (Senile), Keratosis with Squamous Cell Carcinoma. A Clinicopathologic and Histochemical Study. In: Sixth National Cancer Conference Proceedings. Philadelphia: JB Lippincott; 2019. p. 471–88.

7. Anonymous. Indian Pharmacopeia, Volume 1, The Indian Pharmacopoeia Commission, Ghaziabad. 2018; 82-201.

8. Brodland DG, Zitelli JA Surgical Margins for Excision of Primary Cutaneous Squamous Cell Carcinoma. J Am Acad Dermatol 2019-27:241–248.

9. Chandramu C., Manohar RD., Krupadanam DGL., Dashavantha RV.. Isolation, Characterization and Biological Activity of Betulic Acid and Ursolic Acid from Vitex Negundo L. Phytoter. Res. 2018;17:129-34.

10. Chen YT, Zheng T, Holford TR et al. Malignant Melanoma Incidence in Connecticut (United States): Time Trends and Age-Period-Cohort Modeling by Anatomic Site. Cancer Causes Control2022- 5:341–350.

11. T Cjain , Barnetson RS, Zhuang L, et al. Imiquimod induced Regression of Actinic Keratosis Is Associated with Infiltration by T Lymphocytes and Dendritic Cells: A Randomized Controlled Trial. Br J Dermatol 2017;154:72–8.

12. Zambruno G, Reano A, Meissner K, et al. GP37 Expression in Normal and Diseased Human Epidermis: A Marker for Keratinocyte Distribution. Acta Derm Venereol 2018;66:185–92.

13. Yuste Garcia P, Villarejo Campos P, Menendez Rubio JM, et al. Marjolin's Ulcer Arising from a Laparostomy Scar. Int Surg 2006;91:207–10.

14. Zhang C, Cohen JM, Cangiarella JF, et al. Fine-Needle Aspiration of Secondary Neoplasms Involving the Salivary Glands. A Report of 36 Cases. Am J Clin Pathol 2021;113:21–8.

15. Thorn M, Ponten F, Bergstrom R et al Trends in Tumour Characteristics and Survival of Malignant Melanoma –84: A Population-Based Study in Sweden. Br J Cancer 2013-70:743–748.

16. Su, Z. et al. Tumor Promoter TPA Activates Wnt/β-Catenin Signaling in a Casein Kinase 1-Dependent Manner. Proceedings of the National Academy of Sciences of the United States of America. 115 (32),7531 (2020).

17. Van VlotenWA, Hermans J, van DaalWA. Radiationinduced Skin Cancer and Radiodermatitis of the Head and Neck. Cancer 2013;59:411–4.

18. Monica, C. Medical Laboratory Manual for Tropical Countries. 2 Nd Ed., Pp 2019-465-472, Butterworth-Heinemann Ltd, UK.

19. Nijsten TE, Stern RS (2003) Oral Retinoid Use Reduces Cutaneous Squamous Cell Carcinoma Risk in Patients with Psoriasis Treated with Psoralen-UVA: A Nested Cohort Study. J Am Acad Dermatol 2023-49:644–650.

20. Patterson JW, Kao GF, Graham JH, Helwig EB. Bowenoid Papulosis: A Clinicopathologic Study with Ultrastructural Observations. Cancer 2015;57:823–36.

21. Ohnishi Y, Murakami S, Ohtsuka H, et al. Merkel Cell Carcinoma and Multiple Bowen's Disease: Incidental Association or Possible Relationship to Inorganic Arsenic Exposure? J Dermatol 2022;24:310–6.

22. Baoua,M.,Fayn,J. and Bessiere. J.M.. Essais Phytochemique Preliminatary on Some Medicinal Plant in Niger .Plant Med -2018 .10 :251-266.

23. ELGaddal, J.A..Thesis. The Use of Plant Coagulants for Water Treatment in 2011- Rural Sudan, University of Surrey .

24. Barnett CR, Barnett JG, Schwartz RA. Dermatitislike Squamous Cell Carcinoma. Dermatol Surg 2017;30:334–5.

25. Hyre. halim,Weaver, V. M., & Werb, Z. The extracellular matrix: A dynamic niche in cancer progression. The Journal of Cell Biology, 2021-196, 395.

26. J.P. Therrien, R. Drouin, C. Baril, E.A. Drobetsky (2019). Human Cells Compromised for P53 Function Exhibit Defective Global and Transcription-Coupled Nucleotide Excision Repair, Whereas Cells Compromised for pRb Function Are Defective Only in Global Repair. Proc. Natl. Acad. Sci., 96, 15038–15043.

27. Harwood CA, Proby CM,McGregor JM, et al. Clinicopathologic Features of Skin Cancer in Organ Transplant Recipients: A Retrospective Case-Control Series. J Am Acad Dermatol 2006;54:290–300.

28. Frentz G. Grenz Ray-Induced Nonmelanoma Skin Cancer. J Am Acad Dermatol 2018;21:475–8.

29. Chiesa-Vottero A, Dvoretsky PM, Hart WR. Histopathologic Study of Thin Vulvar Squamous Cell Carcinomas and Associated Cutaneous Lesions: A Correlative Study of 48 Tumors in 44 Patients with Analysis of Adjacent Vulvar Intraepithelial Neoplasia Types and Lichen Sclerosus. Am J Surg Pathol 2018;30:310–8.

30. Hoff, M. H., Lyden, D., & Wang, T. C. The Evolution of the Cancer Niche during Multistage Carcinogenesis. Nature Reviews. Cancer,2019, 13, 511–518.