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IN VITRO ANTICANCER AND ANTI-INFLAMMATORY ACTIVITY OF GREEN SYNTHESIZED SELENIUM NANOPARTICLES OF *BALIOSPERMUM MONTANUM* LEAF ON HEPATOCELLULAR CARCINOMA

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ABSTRACT Hepatocellular carcinoma is the major cause of death all over the world and is the fifth most common cancer affecting one million individuals annually. Medicinal plants have immense potential to combat cancer without any known side effects and Selenium is an essential micronutrient that plays a vital role in maintaining good health. In this study Selenium nanoparticles of *Baliospermum montanum* leaf extract [SeNP] were synthesized and characterized using SEM, XRD, UV, and FTIR. Hep G2 cell lines were used to check the anticancer activity by MTT assay and Doxorubicin was used as standard. MTT assay exhibited significantly good results with an IC50 value of 23.38 µg/ml. Two standard methods Proteinase inhibitory action and BSA denaturation assay were used to check the anti-inflammatory activity. The Proteinase inhibitory action exhibited an IC50 value of 698.02 µg/ml and BSA denaturation assay an IC50 value of 440.33 µg/ml. The antioxidant activity of selenium nanoparticles also showed better results by DPPH and ABTS methods. The IC50 value was found to be 72.80 µg/ml for DPPH and 65.73 µg/ml for ABTS method. Overall Selenium nanoparticles of *Baliospermum montanum* leaves exhibited good results for anticancer activity, anti-inflammatory activity, and antioxidant activity against hepatocellular carcinoma and can be a good candidate for therapeutic and pharmaceutical use.

KEYWORDS : Cancer, Selenium nanoparticles, SEM, XRD, FTIR, HepG2.

1. INTRODUCTION

Hepatocellular carcinoma is the major cause of death all over the world and is the fifth most common cancer worldwide affecting one million individuals annually (Ferlay J, 2015). Chemical drugs cause a lot of side effects hence drugs extracted from natural materials like plants are the recent trends. Plants are the principal pharmaceutical stores and are able to generate infinite bioactive compounds and could serve as an alternate source of anticancer agents. Firstly, the biological activity in plants is attributed to the presence of secondary metabolites such as flavonoids, alkaloids, phenols, saponins, steroids, tannins, and terpenoids. This has led to a growing interest in medicinal plants as most pharmaceuticals are becoming plant-derived. Secondly, extracts from medicinal plants have immense potential to combat cancer without any known side effects and they are readily available (M. Greenwell and P.K.S.M. Rahman, 2015). Third, Selenium is an essential trace element in the diet and its nano form has attracted significant attention in biomedical applications, especially in cancer biology (WHO 1973). In this way, nonstop endeavors are being made to explore, recognize and confine the bioactive components and their nanostructures in the plants.

The development of hepatocellular carcinoma (HCC) is caused by a variety of risk factors, including chronic inflammation by the virus, alcohol consumption, and non-alcoholic steatohepatitis. Emerging evidence has notarized inflammation as a critical component of HCC progression. The development of HCC is a multistep process that may originate from liver chronic injury and inflammation to subsequent fibrosis and/or cirrhosis and finally HCC. A large number of studies indicate that chemokines and cytokines are candidates for linking molecules between inflammation and liver cancer. Inhibitors of inflammation for the prevention and overcoming antitumor immunity for the treatment of liver cancer are promising candidates for the future management of patients with HCC (Hong-jin Chenet al, 2018).

Oxidative stress has been investigated for many years as a possible cause of alcoholic liver injury. Furthermore, it has been demonstrated that oxidative stress plays an important role in hepatocarcinogenesis in chronic hepatitis C (CHC). Oxidative stress has been linked to cancer, heart disease, stroke, arthritis, immune deficiency, respiratory diseases, Parkinson's disease, emphysema, and other inflammatory or ischemic conditions. Factors that upsurge the generation of free radicals in the body can be internal, such as inflammation, or external, for example, UV exposure, pollution, and cigarette smoke. A wider range of naturally occurring antioxidants is existing in medicinal plants which are diverse in their composition, physical and chemical properties, and site of action (Sha Li, 2015).

Baliospermum montanum (Muell-Arg) belonging to the family Euphorbiaceae, is principally an important aromatic medicinal plant. It includes 280 genera with 730 species with the largest genus in Euphorbiaceae (Husain *et al.,* 1980: P. Lalitha and P. Gayathiri). Roots, leaves, and seeds of *Baliospermum montanum* are used medicinally and are documented in Asian countries including Nepal, Burma, Malaya, and India. The plant is commonly referred to as Naga danti a threatened medicinal plant (Mali and Wadekar 2008). *Baliospermum montanum* is known for its ethnobotanical and traditional use (Wadekar *et al.,* 2008). The plant is well known for its antioxidant, and anti-inflammatory activity (Kumar Hemant *et al.,* 2011).

In our present study, we have selected an endangered medicinal plant *Baliospermum montanum,* used by folklore, to evaluate its anticancer activity against Hepatocellular carcinoma using HepG2 cell lines as well as its anti-inflammatory and antioxidant activity using different in vitro bioassays. *Baliospermum montanum* leaf extract conjugated with Selenium nanoparticle was used for the study. *Baliospermum montanum* belongs to the family Euphorbiaceae with immense medicinal properties and is a threatened medicinal plant that requires recognition and conservation.

2. MATERIALS AND METHODS

Balliospermum montanum leaves were collected from GKVK, Bangalore in the month of August 2022 (latitude - 12.9716° N and longitude -77.5946° E) as fresh wet leaves, which were then sun-dried, ground, and filtered by sieving to get a fine dry powder and stored in a glass container for further analysis. The plant material was taxonomically identified by Dr. Vasundhara M, Professor, and Head, Department of Horticulture, University of Agricultural Sciences, GKVK, Bengaluru. The voucher specimen with authentication No. 50 was deposited at the Department of Horticulture, University of Agricultural Sciences, GKVK, Bangalore.

The methanolic extract was prepared using the Soxhlet apparatus. A hundred grams of dry powder was mixed with one litre of methanol in a round-bottomed flask and extracted in a Soxhlet apparatus for three consecutive days till a clear solution was obtained. The extracted material was dried and stored in an air-tight glass container and used for further analysis.

2.1 Selenium nanoparticles - Synthesis & characterization

2.1.1 Green synthesis of selenium nanoparticles

Green synthesis of Selenium nanoparticles was initiated by the addition of methanol extract of *B montanum* to selenious acid solution and ascorbic acid as a reducing agent at 40°C. Synthesis of SeNPs was monitored for up to 48 hrs using a UV – Vis spectral analyser with an interval of 2 hrs (Ramamurthy et al. 2013).

2.1.2 Characterization of Selenium nanoparticles

Characterization was done by Ultraviolet-Visible Spectroscopy,

Scanning Electron microscope, X-ray diffraction studies, and Fourier Transform Infrared Spectroscopy (FTIR) analysis (Manikandan 2017).

2.1.3 Ultraviolet-Visible Spectroscopy

Ultraviolet-Visible absorption Spectroscopy is a valuable technique for the characterization of Nanoparticles especially that of metals because they are intensely colored and show absorptions due to surface plasmon oscillations. The absorbance was measured by using Genesys 10S UV-Visible Spectrophotometer. The reduction of selenium ions was monitored by measuring the UV -VIS spectra of the solution at regular intervals on a spectrophotometer.

2.1.4 Scanning Electron Microscope

Scanning Electron Microscope is the most widely used technique to characterize metal nanoparticles which provides information regarding the morphology, distribution, and size of the particle. A scanning electron microscope was used to observe the morphology.

2.1.5 X-ray diffraction studies

The sample was powdered and placed on a Teflon holder. Readings were taken on a Philips XRD (Model:1730/10) CuK- radiation Advanced Eco X-ray powder diffractometer. Readings obtained were analyzed using origin and peak positions.

2.1.6 Fourier Transform Infrared Spectroscopy (FTIR) analysis

Synthesized Selenium nanoparticles (SeNPs) were subjected to FTIR analysis to evaluate the functional groups present on the cell surface that were involved in nanoparticle synthesis. FTIR spectrum was recorded from Bruker (ATR) alpha 2 Germany, 600 to 4,000 cm−1 at a resolution of 2 cm−1 using a Specac Quest Bruker tensor 37 FTIR spectrophotometer.

2.2 Biological Assay of Selenium Nanoparticles

2.2.1 Anticancer Activity of Selenium Nanoparticles

Cytotoxicity test is one of the preferred biological evaluation and screening tests that use tissue cells in vitro to observe cell growth, reproduction, and cell death. Cytotoxicity assays measure the ability of toxic compounds present in the test sample to cause cell damage or cell death. In the present study, Selenium nanoparticles of *Baliospermum montanum* leaf and Hep G2 cell lines were used to evaluate the anticancer activity by the MTT assay method (Mossman, 1983).

Cytotoxicity assay by MTT method

HepG2 cell lines: Cell lines and culture medium

The cell lines were procured from ATCC, stock cells were cultured in DMEM, supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100IU/ml), streptomycin (100µg/ml) in a humidified atmosphere of 5% CO2 at 37°C until confluent growth was obtained. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The viability of the cells was checked and centrifuged. Further, 50,000 cells /well were seeded in a 96-well microtiter plate and incubated for 24 hrs at 37°C, 5% CO2 incubator. All the reagents DMEM, FBS, PenStrep, and Trypsin were procured from Invitrogen.

Preparation of test solutions:

For cytotoxicity studies, 32mg/ml stocks were prepared and serial two-fold dilutions were prepared from 320µg/ml to 10µg/ml using DMEM plain media for treatment.

Procedure:

The monolayer cell culture was trypsinized and the cell count was adjusted to 1 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96-well microtiter plate, 100µl of the diluted cell suspension (50,000 cells/well) was added. After 24 hrs of incubation, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with the medium. 100µl of different test concentrations of test samples [10 µg/ml to 320 µg/ml], control, and standard were added to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24 hrs in a 5% CO2 atmosphere. After incubation, the test solutions in the wells were discarded and 100 µl of MTT (6 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 hrs at 37°C in a 5% CO2 atmosphere. The supernatant was removed and 100µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590nm. The percentage growth inhibition was calculated using the formula and the concentration of test sample needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line.

***2.2.2* Anti-Inflammatory Activity Of Selenium Nanoparticles**

The anti-inflammatory activity of Selenium particles was evaluated using two in vitro-based assays: Proteinase Inhibition and BSA Denaturation Assay**.**

***2.2.2.1* Proteinase Inhibitory Assay**

The assay was carried out as per the method of Oyedepo and Femurewa, 1995 with slight modifications. 2 mL of reaction mixture containing 0.06 mg proteinase, 1 mL of 20 mM Tris HCl buffer (pH 7.4), and 1 mL of different concentrations of test sample ranging from 0.1 mg – 0.5 mg were taken in a series of test tubes and incubated at 37° C for 5 minutes. Similarly, different concentrations of standard diclofenac were also mixed with the reaction mixture and incubated at 37° C for 5 minutes. Then, 1 mL of 0.8% casein was added to all the tubes and incubated at 37° C for 20 minutes. Later, 2 mL of 70% Perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm **(**Oyedepo and Femurewa, 1995). The percentage of inhibition was calculated.

***2.2.2.2* BSA Denaturation Assay:**

The solution containing 1 ml of different concentrations of Selenium nanoparticles ranging from 0.1 – 0.5 mg was mixed with 1 ml of 0.1% Bovine Serum Albumin (BSA) solution and incubated at 37**º**C for 15 minutes. Similarly, different concentrations of standard diclofenac were also mixed with BSA and incubated at 37° C for 15 minutes. Denaturation was induced by keeping the reaction mixture at 60**º**C in the water bath for 10 minutes. After cooling, the turbidity was measured spectrophotometrically at 600 nm. The percentage of inhibition was calculated (Oyedepo and Femurewa,1995).

***2.2.3* Antioxidant activity of Selenium nanoparticles**

The antioxidant activity of Selenium nanoparticles was carried out by two important standard methods DPPH and ABTS method.

***2.2.3.1* DPPH method**

The radical scavenging activity was studied using 1-1-diphenyl-2-picrylhydrazyl (DPPH). Two ml of various dilutions (10 µg/ml -50 µg/ml) of Selenium nanoparticles were mixed with 5 ml of 0.1 mM methanol DPPH solution and incubated at 37**º**C for 30 min. The wavelength of maximum absorbance of DPPH was measured at 517 nm using a spectrophotometer. All measurements were performed in triplicates. The percentage of free radical scavenging activity of each concentration was calculated **(**Blois, 1958).

***2.2.3.2* ABTS method**

The dye 2, 2, Azino Bis-3-ethylbenzothiazoline-6-sulphonate (ABTS) radical cation decoloration assays were used for the determination of antioxidant activity. 1 ml of diluted ABTS solution was added to the Selenium nanoparticles of different aliquots ((10 µg/ml -50 µg/ml)). Absorbance was measured at 734 nm after 30 min. of incubation at room temperature. The capacity of free radical scavenging was determined **(**Blois, 1958).

3. RESULTS AND DISCUSSION

3.1 Extraction of plant

Extraction was conducted using methanol as the solvent which showed maximum extraction from the leaf powder. Phytochemical analysis showed the presence of alkaloids, carbohydrates, protein, saponins, cardiac glycosides, phenol, steroid, terpenoids, glycoside, tannin, flavonoids, and resins present in the extracts (Lalitha and Gayathiri, 2013).

3.2 Synthesis of Selenium nanoparticles from *B. montanum* methanol extract & characterization

3.2.1 Green synthesis of Selenium nanoparticles and characterization

The development of red color indicated the formation of selenium nanoparticles due to the reduction of selenium ions to elemental selenium in the medium after 48 hrs. Synthesized Selenium nanoparticles (SNP) were characterized and confirmed by UV- Vis Spectrophotometer, scanning electron microscope, X-ray powdered diffraction (XRD) studies, and Fourier transform infrared spectroscopy (FTIR).

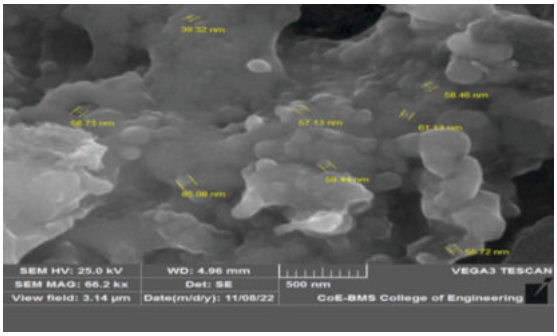

Fig. 1. Results of SEM showing the morphology and size of the Selenium nanoparticle.

UV- Vis spectrophotometer Spectral analysis revealed the peak position at 263 nm representing the formation of selenium after 48 hrs. The particle size, shape, and nature were studied by SEM analysis and were found to be oval, porous, and Crystalline. The size was below 100 nm (39.39, 55.72, 57.13,58. 46 58.73, 59.44, 83. 90.7nm). (Fig. 1).

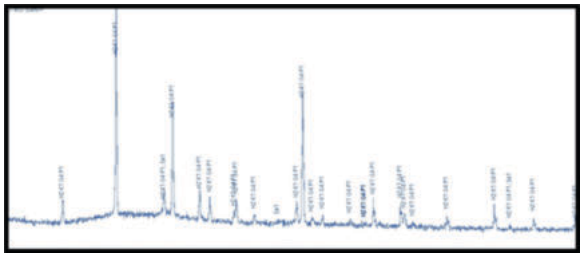

Figure 2. Chromatogram of X-ray Diffraction studies showing the peaks.

X-ray diffraction studies showed characteristic peaks at 2θ values corresponding to 30 and 40.3. Peak confirms the presence of selenium Nanoparticles in the medium. Studies also confirm the crystalline nature of the nanoparticles (Fig. 2). FTIR spectrum was recorded between 4,000 and 600 cm^{-1}.

FTIR spectrograph of Selenium nanoparticles synthesized from *B montanum* exhibited major peaks between 1300 and 2400 cm^{-1} (Fig 3). Peaks correspond to N-H, O-H, C=O, NO2, N-H, and C=C stretching and the presence of functional groups amines, alcohols, carboxyl, nitroalkanes, amines, aromatics, and alkenes.

3.3 Biological Assay of Selenium Nanoparticles

Synthesized Nanoparticles were checked for their biological assays such as anticancer, anti-inflammatory, and antioxidant activity.

3.3.1 Anticancer Activity of Selenium Nanoparticles

Cytotoxicity assay by MTT method using HepG2 cell lines

Cytotoxicity of Selenium nanoparticles was checked by MTT Assay against HepG2 cell lines. Doxorubicin was taken as standard. The test sample showed a percentage inhibition ranging between 12.03 µg/ml to 70.08 µg/ml and an IC50 value of 94.33 µg/ml inhibition (Tab.1). Standard Doxorubicin showed a percentage of inhibition ranging between 22.70 µg/ml and 86.34 µg/ml and an IC50 value of 23.38 µg/ml inhibition in HepG2 cells (Tab.2). The study shows that the extracts of Selenium nanoparticles were cytotoxic to HepG2 cell lines and a positive test for MTT assay. The percentage of inhibition steadily increased with an increase in the concentration of the Selenium extract. The cytotoxic effect may be due to the presence of phytochemicals such as flavonoids, terpenoids, steroids, and phenolic compounds present in the plant extract. Furthermore, the study shows that the extract can be used for the treatment of HCC.

Table 1. MTT Assay result showing the percentage of inhibition and IC 50 value of Hep G2 cells by Selenium nanoparticles.

Sample	Con µg/ml	OD@590nm	% Inhibition	IC50 in µg/ml
Control	0	0.615 ± 0.003	0.00	
Sample	10	0.541 ± 0.022	12.03	94.33
	20	0.478 ± 0.0037	22.28	
	40	0.402 ± 0.015	34.63	
	80	0.366 ± 0.038	40.49	
	160	0.271 ± 0.04	55.93	
	320	0.184 ± 0.022	70.08	

Table 2. MTT Assay result showing the percentage of inhibition and IC 50 value of HepG2 cells by standard Doxorubicin.

Sample	Con µg/ml	OD@590nm	% Inhibition	IC50 in µM
Control	0	0.615 ± 0.003	0.00	23.38
Doxorubicin	3.13	0.475 ± 0.051	22.70	
	6.25	0.389 ± 0.025	36.75	
	12.5	0.356 ± 0.043	42.11	
	25	0.241 ± 0.033	60.81	
	50	0.152 ± 0.056	75.28	
	100	0.084 ± 0.06	86.34	

Overall, the synthesized nanoparticles from the Baliospermum montanum plant have shown good results as compared to standard doxorubicin and because of its long antiquity of medicinal use for other ailments and various cancer treatments, this plant can be considered as a potential and promising alternate agent in the medication of hepatocellular carcinoma.

All the categories of cancers have developed various mechanisms to escape regulated progress and evade apoptosis. Hence the use of newer and different plants which contain several components having diverse probable intracellular targets may offer preventive remedies in combination with other treatments. Additionally, most of the medicinal and therapeutic plants tested have a long history of medicinal use and are nontoxic, thus having the potential to act as promising medications. Our current study outcomes may contribute towards the authentication of this rare and indigenous plant as a potentially effective chemotherapeutic agent in treating HCC.

3.3.2 Anti-inflammatory Activity of Selenium Nanoparticles

The anti-inflammatory activity of Selenium nanoparticles was conducted by two Standard methods: Proteinase inhibition method and BSA Denaturation assay

3.3.2.1 Proteinase Inhibition Method

The anti-inflammatory activity of the Selenium extract was performed employing one of the standard methods, the Proteinase inhibition method. To evaluate the efficiency of the Selenium nanoparticles, different concentrations of the Selenium extracts ranging between 100 µg to 500 µg were used in the study. Diclofenac was used as standard. The percentage of inhibition was checked for both the test sample and diclofenac.

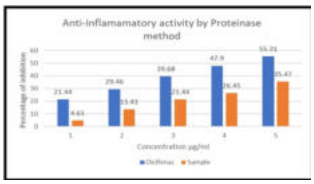

Figure 4. Anti-inflammatory activity of Selenium nanoparticles and Standard by Proteinase Inhibition method

The percentage of inhibition for the sample ranged between 4.61 µg and 35.47 µg. And standard between 21.44 µg and 55.31 µg. The maximum percentage inhibition was observed in the highest extract concentration. As expected, the inhibitory activity was found to be concentration dependent in both test samples as well as standard. IC 50 value was calculated and was found to be 698.02 µg for the plant sample and 430.35 µg for standard Diclofenac by proteinase inhibitory action showing good anti-inflammatory action by the plant (Figure 4).

Proteinases are proteolytic enzymes that break down protein. In the study, the results show that the extract possesses certain proteolytic enzymes capable of degrading the protein in the reaction mixture. Besides this, the study also states the inhibitory activity of the selenium particles which has the capacity to degrade the proteins present in the cancer cells, thus inhibiting the growth and progression of HCC.

3.3.2.2 BSA Denaturation Assay

The anti-inflammatory activity was also evaluated by the BSA denaturation method. The percentage of inhibition ranged between 3.28 µg and 57.38 µg for the plant sample and 8.20 µg and 81.97 µg for

the standard. The results of this assay showed an IC 50 value of 440.33 µg for the Selenium nanoparticles and 332.21 µg for Standard Diclofenac. Results showed good anti-inflammatory action by the plant (Figure 5).

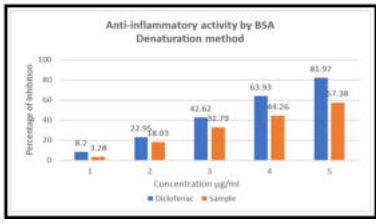

Figure 5. Anti-inflammatory activity of Selenium nanoparticles and standard by BSA Denaturation method.

3.3.3 Anti-Oxidant Activity of Selenium Nanoparticles

The antioxidant activity of selenium nanoparticles was conducted by two standard methods DPPH and ABTS method.

3.3.3.1 Antioxidant activity of Sample by DPPH method

DPPH radical scavenging is an accepted mechanism for evaluating the antioxidant activity of the plant extracts, where violet-colored DPPH is reduced to yellow-colored diphenylpicryl hydrazine upon adding the plant extract. The amount of scavenging can be determined by measuring absorbance at 517 nm with a spectrophotometer.

In the present study, the DPPH radical scavenging activity of different concentrations of Selenium nanoparticles synthesized from methanolic extract of *Baliospermum montanum* exhibited good antioxidant activity. The evaluation showed inhibition activity ranging between 10.19 µg and 36.19 µg for the sample. The Standard ranged between 14.73 µg and 77.70 µg. IC50 values were calculated using the graph and were found to be 72.80 µg and 30.10 µg for sample and standard respectively (Fig 6). Our study results show that the plant possesses compounds with scavenging ability. Previous studies have also shown that the plant withholds different phytochemicals with an enormous amount of antioxidant properties. Our present study is in alliance with the earlier studies.

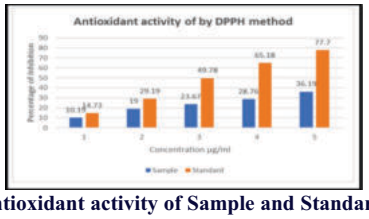

Figure 6. Antioxidant activity of Sample and Standard by DPPH method

3.3.3.2 Antioxidant activity of Sample by ABTS method

ABTS assay is based on the generation of a blue/green ABTS that can be reduced by the antioxidants and measured spectroscopically. ABTS assay was used to determine the antioxidant potential of selenium extracts by measuring their ability to act as free radical scavengers. Ascorbic acid was taken as standard. An increase in the Selenium extract concentration resulted in a simultaneous increase in the ABTS scavenging activity. The scavenging activity was measured in terms of percentage inhibition. The percentage inhibition of the Selenium particles ranged between 6.05 µg and 35.27 µg and the standard between 28.99 µg and 67.35 µg. IC value was found to be 65.73 µg for the sample and 31.52 µg for the standard (Fig 7). The assessment indicated the presence of antioxidants in the plant extract. Antioxidant potentials of plants were more strongly reported previously by many researchers and correlated the activity to the presence of phenolic compounds and flavonoid content in the plants(Kim et al., 2003). Our findings taken together also suggest that the plant possess antioxidant and can be used in conjunction with the treatment of HCC.

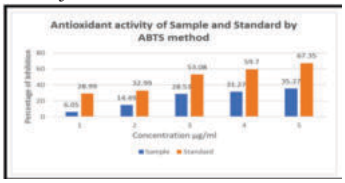

Figure 7. Antioxidant activity of Sample and Standard by ABTS method

STATISTICALANALYSIS

Statistical analysis is a scientific data analysis tool that helps to draw meaningful conclusions from raw and unstructured data. In our present study, all the experiments were conducted in triplicates and the data were presented as mean standard deviation from three experiments. Statistical analysis was carried out by ANOVA and the p-value was found to be below 0.05, hence considered statistically significant.

4. CONCLUSION

Nanotechnology is one of the interdisciplinary areas of research with valuable commercial applications and has been extensively studied because of its unique physical properties, chemical activity, and potential applications in the field of medicine. Metal nanoparticles are found to be potential therapeutic alternatives for the treatment of various diseases including cancer. For the first time, the synthesis of selenium nanoparticles using *Baliosprmum montanum* leaf was carried out. Selenium is the most widely used material due to its biomedical and antimicrobial and anti-cancerous applications among the metal particles such as gold, silver, iron, palladium, and zinc. According to the literature and the results of the present study reveals that the major constituents present in the extract were responsible for the therapeutic property of *B. montanum* hence, the plant can be used commercially by pharmaceutical companies for the preparation of reliable drugs which can cure HCC. Further research is needed to separate/isolate the active ingredients that are responsible for curing the disease. Moreover, in vivo studies are also required. In conclusion, our study strongly supports the pharmaceutical and medical importance of this potential red-listed plant *B. montanum* which requires conservation.

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CONFLICT OF INTEREST

There is no conflict of interest.

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