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Phytochemical Study and Anticancer Activity of *Heterodermia boryi*

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A B S T R A C T

Lichens exhibit various biological properties due to the presence of secondary metabolites composed of alkaloids, flavonoids, phenols and terpenoids. *Heterodermia boryi* is a lichen species with various chemically important biota but remains largely unexplored. The objective of this study is to investigate the phytochemical constituents and anticancer properties of *Heterodermia boryi* isolated from the hills of Kodaikanal, India. *Heterodermia boryi* exhibited significant anticancer properties against human lung, liver and breast cancer cell lines. Further studies, should be carried out to explore the potential biological activities of *Heterodermia boryi*.

Introduction

Novel bioactive compounds are the need of the hour due to increase in the number of infectious diseases and their subsequent antimicrobial resistance (Alurappa *et al.*, 2018). Lichens are symbionts and occur universally in various substrates.

Lichens and its metabolites are of biological interest due to their antioxidant, anti-inflammatory, anti-cancer and antimicrobial properties (Prashith *et al.*, 2018; Devi *et al.*, 2012 and Srinivasan *et al.*, 2010). *Heterodermia boryi* species has been known to exhibit antibacterial and anthelmintic properties as a result of the existence of its metabolities which are composed of alkaloids, flavonoids, aliphats, aromats and terpenes (Senthil Prabhu and Sudha, 2016 and Senthil Prabhu and Sudha, 2015). This *in vitro* research prospects the phytochemical and anti-cancer properties of *Heterodermia boryi* isolated from the hills of Kodaikanal, India.

Materials and Methods

Qualitative Phytochemical Analysis

The presence of various phytochemicals was screened in aqueous, ethanol, methanol, acetone and

ethyl acetate lichen extracts. The extracts were exposed to different chemical tests for the identification of different phytoconstituents including the alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, glycosides, phenols, carbohydrates and proteinsusing standard procedures.

Alkaloids

Meyar's reagent (potassium iodide)

In 60 ml of Distilled water, 1.3 gm of Mercuric Chloride and in 10ml of Distilled water 5.0 gm of Potassium Iodide was dissolved. The two solutions were mixed and diluted to 100 ml with distilled water. To 1.0 ml of lichen extract, drops of Meyar's reagent were added. White/Pale-yellow precipitate formed indicated presence of alkaloids.

Phenols

Ferric chloride test

1.0 ml of lichen extract were added 2.0 ml of distilled water and a few drops of 10 % aqueous FeCl3 solution was added. Formation of blue or green colour indicated the presence of phenols.

Flavanoids

In the test tubes containing 0.5 ml of lichen extract, 5-10 drops of dilute HCl and small piece of magnesium was added and the solution was boiled for a few minutes. Due to presence of flavonoids, reddish pink or dirty brown colour was produced.

Tannins

Ferric chloride test

1 ml of lichen sample extract, 5 % aqueous FeCl3 solution was added in drops. After the addition of a few ml of dilute H2SO4 the bluish black colour precipitate turned to a yellowish-brown precipitate.

Saponins

In the test tube containing about 5.0 ml of lichen extract, a drop of sodium bicarbonate solution was added and the contents were mixed and shaken vigorously. The test tube was kept undisturbed for 3 minutes. A honey comb like froth was formed and it indicated the saponins presence.

Terpenoids

Salkwski reaction

To 5.0 ml of lichen extract, 2.0 ml of chloroform was added and mixed. Concentrated H_2SO_4 (3.0 ml) was carefully added to form a layer following it. A reddish-brown coloration formed at the inter phase shows the presence of terpenoids as a positive result.

Steroids

Libermann-Burchard's test

1.0 ml of lichen extract and 1.0 ml of Concentrated H_2SO_4 was mixed carefully. To this approximately 2.0 ml of acetic anhydride solution was added. A greenish colour developed and turned blue indicating the existence of steroids.

Carbohydrates

Benedict's test

173 g of sodium citrate and 100 g of sodium carbonate was dissolved in 500 ml of water. To this solution 17.3 g of copper sulphate dissolved in 100 ml of water was added. To 0.5 ml of the lichen extract, 5.0 ml of Benedict's reagent was added and boiled for 5 minutes. Formation of a bluish green colour showed the presence of carbohydrates.

Glycosides

A little amount of lichen extract added in 1.0 ml of water, dissolved and then an aqueous sodium hydroxide solution was added. Formation of a yellow colour indicated that glycosides are present.

Aminoacids and Proteins

Biuret's test

1.0 ml of lichen extract, 5-8 drops of 5 % sodium hydroxide solution were added, followed by one or two drops of 1 % copper sulphate. Formation of pink or purple colour indicated the presence of amino acids and Proteins (Peach and Tracey, 1955 and Raaman, 2006).

Quantitative Analysis of Phytochemicals

Estimation of Total Alkaloids

10 mg of lichen material was homogenized in a mortar and pestle and 20ml of methanol: ammonia (68:2) was added. After 24hrs fresh methanolic ammonia was added to decanted ammoniacal solution. The procedure was replicated thrice and the extracts were pooled. The extracts were evaporated using a flash evaporator.

The residue was treated with 1N HCl and kept overnight. The acidic solution was extracted with 20ml of $CHCl_3$ thrice and the organic layers were pooled and evaporated to dryness. Sodium hydroxide was added to acidic solution and the pH was adjusted to12.

This extract was treated with $CHCl_3$ (20ml) thrice, and the layers were pooled and evaporated to dryness. The fraction that contained ajmalicine and serpentine was weighed (Harborne, 1973).

Estimation of Total Phenol

Extraction

0.5 g of lichen sample was weighed and ground with a mortar and pestle in 5 ml of ethanol. It was centrifuged at 2000 rpm for 10 min and the supernatant was collected in 50 ml volumetric flask. Then the residue was evaporated and dissolved in known volume of water and used for the assay.

Procedure

An aliquot of the lichen sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath exactly for 1 min. It was then cooled and the absorbance was measured at 650 nm using spectrophotometer against the reagent blank.

A calibration curve was constructed with different concentrations of gallic acid (0.01-0.1 mM) as a standard and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract (Singleton and Rossi, 1965).

Estimation of Total Flavonoids

Procedure

A volume of 0.5 ml of 2% of $AlCl_3$ in ethanol solution was added to 0.5 ml of sample. After one hour incubation at room temperature, a yellow color was developed. This was measured at 420 nm with UV-Visible spectrophotometer.

A standard graph was prepared using the quercetin as a standard and the total flavonoid content was expressed as quercetin equivalent (mg/g) (Ordonez *et al.,* 2006).

Estimation of Total Terpenoid Content

To 1 mL of the lichen extract, 3 mL of chloroform was added. The sample mixture was thoroughly mixed and left for 3 min and then 200 µl of concentrated sulfuric acid (H_2SO_4) was added. It was then incubated at room temperature for 2 hours in dark condition during which a reddish-brown precipitate was formed.

The supernatant from the reaction mixture was decanted carefully without disturbing the precipitate. 3 mL of 95% (v/v) methanol was added and mixed thoroughly until all the precipitate dissolved in

methanol completely. The absorbance was read at 538 nm using UV/visible spectrophotometer (Ghorai *et al.*, 2012).

MTT Assay

Cell lines

The human liver cancer cell line (HepG2), lung cancer cell line (A549), human breast cancer cell line (MCF 7) and normal cell line (Vero) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passage weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions. The resulting density of $1x10^5$ cells/ml was achieved by diluting with medium containing 5% FBS and the viable cells were counted using a hemocytometer. Cell suspension of 100 µl per well were seeded into 96-well plates with a density of 10,000 cells/well. The wells were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity to allow cell attachment. After 24 hours the cells were incubated with serially concentrated test samples. The cells were originally dissolved in dimethylsulfoxide (DMSO). An aliquot of the Lichen sample solution was diluted two times with serum free medium forthe desired final maximum test concentration. Additional four serial dilutions were prepared to provide five sample concentrations in total. These different sample dilutions in aliquots of 100 µl were added to the respective wells, containing 100 µl of medium. This resulted in the required final sample concentration. The plates are incubated for an additional 48 hours at 37°C, 5% CO_2, 95% air and a relative humidity of 100%. The medium without samples served as control while triplicates were maintained for all concentrations.

After 48 hours of incubation, 15µl of MTT (5mg/ml) dissolved in phosphate buffered saline was added to each well followed by incubation at 37°C for 4hrs.The medium along with MTT was flicked off and the formazan crystals formed were solubilised in 100µl of DMSO and then the absorbance was measured at 570 nm using micro plate reader (Mosmann, 1983; Monks *et al.*, 1991).

Results and Discussion

Qualitative phytochemical analysis revealed that the phytochemicals were more evident in ethanol, methanol and acetone when compared to aqueous and ethyl acetate extracts (Table 1). Quantitative analysis of the extract showed the presence of alkaloids, phenols, flavonoids and terpenoids (Table 2). The MTT assay showed that the lichen extract markedly inhibited proliferation of $HepG_2$, A549 and MCF 7 cells in a dose-dependent manner (Table 3, 4, 5, 6, 7, 8, 9 and 10).

Lichens have a vast importance in medicine based on the unique and varied biologically phytoconstituents contained in it (Priya Srivastava *et al.*, 2013). Five different solvent extracts of *Heterodermia boryi* were prepared in water, ethanol, methanol, acetone and ethyl acetate. The chemical tests performed revealed the existence of several phytochemicals. Alkaloids were more in acetone, ethanol and methanol extracts. Phenols and flavonoids in the ethanol, methanol and acetone extracts were well evident. Tannins, saponins and steroids seemed to be absent in few extracts while carbohydrates, glycosides are observed in all extracts. Amino acids and proteins were significantly observed in all extracts. Terpenoids were observed in ethanol and methanol but not in other extracts. The constituents in various extracts interact with each other in synergism or antagonism to exhibit antimicrobial, anti-oxidant and anti-cancer activities (Ristić *et al.*, 2016). The quantitative phytochemical analysis of the lichen extract showed the presence of alkaloids, phenols, flavonoids and terpenoids. The total content of alkaloids was 5.55mg/g.

Table.1 Qualitative analysis of phytoconstituents present in different solvent extracts of lichen samples

Phytochemicals	Aqueous	Ethanol	Methanol	Ethyl acetate	Acetone
Alkaloids	+	++	++	+	++
Phenols	++	+++	+++	+	+++
Flavonoids	++	+++	+++	+	++
Tannins	+	++	+	++	+
Saponins	-	+	+	+	-
Terpenoids	-	+++	+++	+	+
Steroids	-	++	+	-	+
Carbohydrates	+	++	+	+	+
Glycosides	+	++	+	+	+
Amino acids	+	+++	+++	++	+++
Proteins	+	+++	+++	++	+++

+ → present in small concentration; ++ → present in moderately high concentration; +++ → present in very high concentration; -- → absent

Table.2 Quantitative analysis of ethanol extract for primary and secondary metabolites present

Primary and Secondary metabolites	Ethanol extract
Alkaloids (mg/g sample)	5.55 ± 0.50
Total phenols (mg/g sample)	3.30 ± 0.15
Flavonoids (mg/g sample)	2.88 ± 0.55
Terpenoids (mg/g sample)	4.50 ± 0.10

Values are expressed as mean ± SD (n=3)

Table.3 Liver cancer cell line (HepG2) concentrations

Concentration	18.75 µg	37.5 µg	75 µg	150 µg	300 µg	Control
ABS	0.421	0.364	0.288	0.215	0.176	0.507
	0.423	0.365	0.285	0.217	0.174	0.509
	0.42	0.362	0.287	0.213	0.177	0.508
Avg	0.421333	0.363667	0.286667	0.215	0.175667	0.508

Table.4 Liver cancer cell line (HepG2) cell inhibition

Concentration (µg/ml)	% Cell inhibition
18.75	17.12
37.5	28.54
75	43.7
150	57.67
300	65.55

Table.5 Lung cancer cell line (A549) concentrations

Concentration	18.75 µg	37.5 µg	75 µg	150 µg	300 µg	Control
ABS	0.457	0.372	0.289	0.211	0.185	0.521
	0.455	0.371	0.292	0.215	0.187	0.522
	0.456	0.373	0.293	0.214	0.186	0.523
Avg	0.456	0.372	0.291333	0.213333	0.186	0.522

Table.6 Lung cancer cell line (A549) cell inhibition

Concentration (µg/ml)	% Cell inhibition
18.75	12.64
37.5	28.73
75	44.25
150	59.19
300	64.36

Table.7 Human breast cancer cell line (MCF 7) Concentrations

Concentration	18.75 µg	37.5 µg	75 µg	150 µg	300 µg	Control
ABS	0.478	0.413	0.361	0.321	0.241	0.539
	0.478	0.415	0.363	0.323	0.243	0.537
	0.479	0.414	0.363	0.325	0.243	0.546
Avg	0.478333	0.414	0.362333	0.323	0.242333	0.540667

Table.8 Human breast cancer cell line (MCF 7) cell inhibition

Concentration (µg/ml)	% Cell inhibition
18.75	11.48
37.5	23.33
75	32.96
150	40.18
300	55.18

Table.9 Normal cell line (Vero) Concentration

Concentration	12.5 µg	25 µg	50 µg	100 µg	200 µg	Control
ABS	0.397	0.392	0.391	0.385	0.381	0.399
	0.397	0.392	0.39	0.384	0.382	0.398
	0.395	0.394	0.388	0.385	0.382	0.399
Avg	0.396333	0.392667	0.389667	0.384667	0.381667	0.398667

Table.10 Normal cell line (Vero) cell growth

Concentration (µg/ml)	% Cell Growth
12.5	99.41471572
25	98.49498328
50	97.74247492
100	96.48829431
200	95.73578595

Fig.1

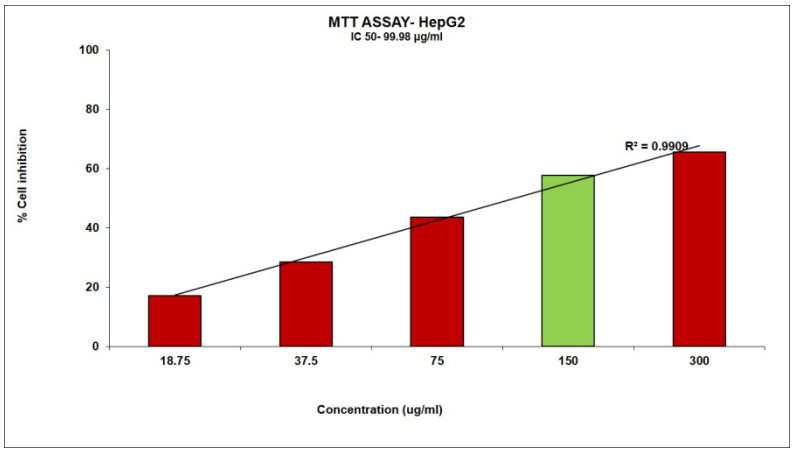


Fig.2

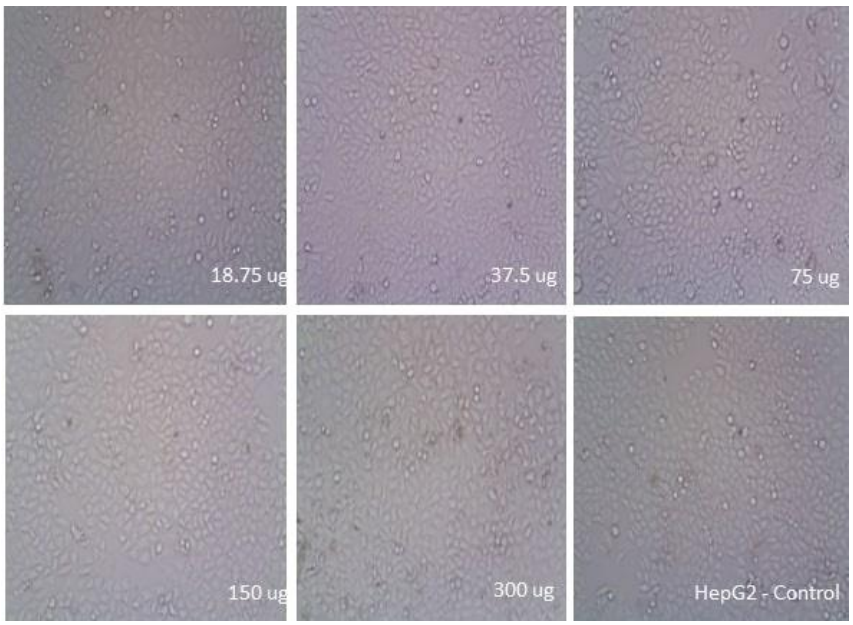


Fig.3

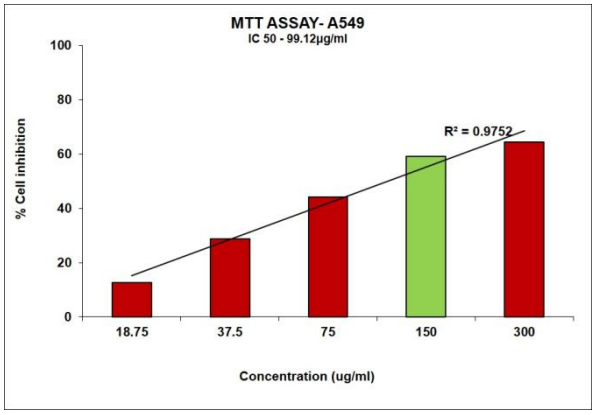


Fig.4

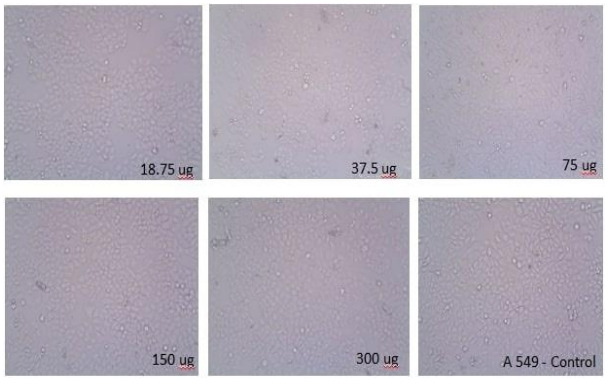


Fig.5

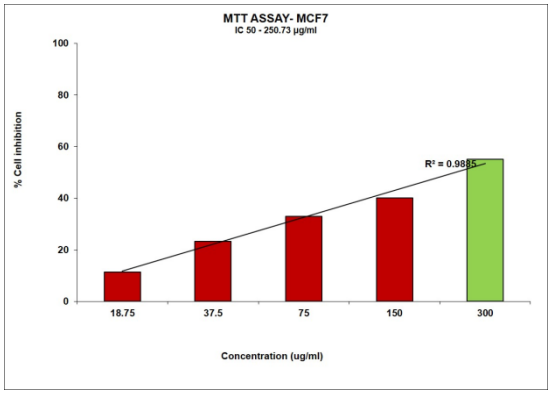


Fig.6

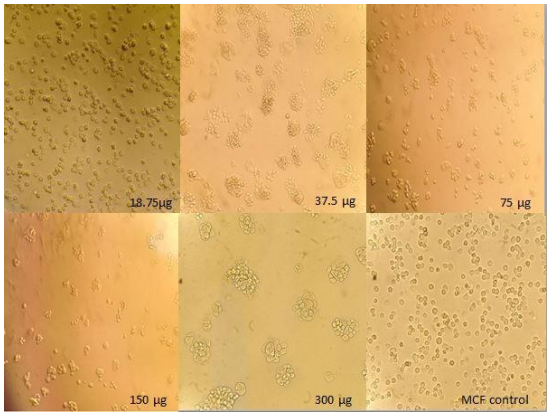


Fig.7

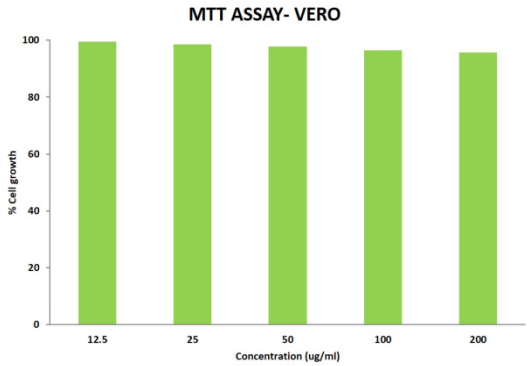
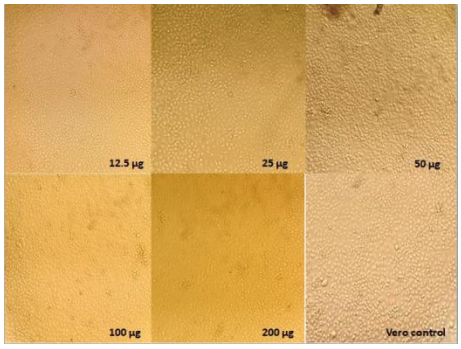


Fig.8



Alkaloids which are nitrogen rich metabolites are poorly understood (Dembitsky and Valery, 2015). Kuklev *et al.,* (2016) observed that alkaloids showed effective cytotoxicity against MCF 7 cell line and murine P815 mastocytomia cell line (Kuklev *et al.,* 2016). The blue coloured complex (molybdenum blue)formed due to presence of phenols was colorimetrically estimated at 650nm.

The blue developed because of Phenols reacting with phosphomolybdic acid of Folin-Ciocalteau reagent in an alkaline medium. The total content of phenols was 3.30mg/g. Simple phenols associated with secondary lichen metabolites are formed by

acetate-malonate pathway (Bombuwela *et al.,* 2010). Several studies have found a positive correlation between the total content of phenols and the antioxidant activity. The protective effective against chronic diseases increased with an increase in the total phenolic content. Phenols also serve as free radical terminators (Noura Aoussar *et al.,* 2020; Kosani´c *et al.,* 2013 and Nguyen and Dinh, 2019).

Flavonoids reacts with aluminium chloride in ethanolic solution forms a yellow color which was read colorimetrically at 420 nm. The total flavonoid content estimated was 2.88 mg/g. Aoussar observed no significant correlation between the flavonoid content in lichen extract and antioxidant activity (Noura Aoussar *et al.,* 2020). The total content of terpenoids were 4.50 mg/g. Terpenoids are produced in lichens by mevalonic acid pathway and have been observed as antioxidant, anticancer, and immunosuppressive agents (Dembitsky and Valery, 2015; Elkhateeb and Daba, 2020).

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water-soluble tetrazolium salt. Succinate-dehydrogenase is a mitochondrial enzyme present in the living cells. It converts the MTT to an insoluble purple formazan by cleaving the tetrazolium ring.It is evident that the amount of formazan produced is directly corelated to the count of viable cells. Antiproliferative effects of ethanol extracts were evaluated by MTT assay against MCF-7 (human breast cancer) HepG2 (human hepatocellular carcinoma) and A549 (adenocarcinomic human alveolar basal epithelial cells) cell lines. The HepG2 cell line showed maximum vulnerability against the lichen extract (Figure 1 and 2). Lung cancer cell line (A549) showed nearly equal vulnerability against the ethanol extract with 12.64% and 64.36% cell inhibition at 18.75 µg/ml and 300 µg/ml Concentrations respectively (Figure 3 and 4). The % growth with treatment of the lichen extract at lowest Concentration level 18.75 µg/ml was 17.12 and at highest Concentration level 300 µg/ml was 65.55. The Cell inhibition percentage of the MCF-7, human breast cancer cell line (Figure 5 and 6) compared to the other cell lines showed much less vulnerability when treated with the lichen extract. At low concentration level of 18.75 µg/ml, the cell inhibition percentage was 11.48 and at high concentration level of 300 µg/ml, it was found to be 55.18%. The loss of viability of the dying cancer cells can be evidenced by the morphological changes scrutinized by microscopy. Different levels of cytotoxicity like cell shrinkage, aggregation and cell death could be observed at different concentration of the lichen extracts. In contrast the Vero cell line exhibited growth and cell inhibition percentage was negligible (Figure 7 and 8). These data suggests that the extracts are more toxic to cancer cells than normal cells. Considering the activity of the extracts, the lichen sample could be considered as potential anticancer drugs.

In accordance with our study, several authors have reported anticancer properties of lichen metabolites against human liver, lung and breast cancer (Kumar *et al.,* 2014; Fernández-Moriano *et al.,* 2015; Ozturk *et al.,* 2021 and Zhou *et al.,* 2017). Lichens exert their anticancer property by various biological activities such as an antioxidant, anti-proliferative cytotoxic, pro-apoptotic, anti-invasive, anti-migrative, and overall anti-tumorigenic abilities (Solárová *et al.,* 2020).

Heterodermia boryi is a largely unexplored species with potential biological activities. Phytochemical and anticancer studies involving the lichen *Heterodermia boryi* have not been previously reported in literature. This is the first study in which metabolic constituents and the anticancer properties of *Heterodermia boryi* have been investigated. Significant cytotoxicity was observed against HepG2 (liver cancer), A549 (lung cancer) and MCF-7 (human breast cancer). Further studies exploring the individual metabolites and constituents should be carried out to utilize the pharmacological and biological properties of this unexplored lichen species.

Conflict of Interest

The authors declare that they have no conflict of interest.

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