In-vitro study of antiproliferative and anthelminthic property of medicinal plants of Kokrajhar, Assam

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Abstract

People living in far-flung areas of the world, especially ethnic tribal people practice traditional medicine as the first choice of disease treatment. The present study investigates the antioxidant, cytotoxicity, and anthelmintic activity of four medicinal plants traditionally used by tribal communities of Bodoland Region of Assam. Total phenolic and flavonoid content was estimated following spectrophotometry method. Antioxidant activity was measured by total antioxidant assay, FRAP, DPPH, ABTS, and TBARS assay. Antiproliferative and apoptosis-inducing activity of plants were carried out in DL cells. Cells were treated for 24 h with different doses of plant extracts. Furthermore, anthelmintic study was carried out by treating the helminth parasite at different doses of plant extracts. Phytochemical and antioxidant study showed rich TPC, TFC, and free radical scavenging activity in *H. japonicum* and *H. sibthorpioides*. Both the antiproliferative and anthelmintic activity showed a dose-dependent efficacy in all the plants. *H. japonicum* showed the strongest anthelmintic activity with LC_{50} 212.78 µg/mL followed by *H. sibthorpioides* (5.36 mg/mL), *C. halicacabum* (13.40 mg/mL), and *A. scholaris* (18.40 mg/mL). On the other hand, *H. sibthorpioides* showed stronger antiproliferative and apoptosis-inducing activity compared to other plants. The study observed a positive correlation between the antioxidant property and antiproliferative and anthelmintic activities of the plants. We, therefore, conclude that the secondary metabolites along with antioxidant molecules may have combined effects contributing the antiproliferative and anthelmintic activity of the plants.

Introduction

Helminths are a group of eukaryotic organisms that affects millions of people worldwide and also cause severe economic loss to the livestock industry (Qamar et al. 2011). Among the major helminthiasis, soil-transmitted helminthiasis and schistosomiasis represents more than 1 billion people worldwide, mainly in poor economic countries of the world (James et al. 2018). It has been estimated that the mass deworming program of helminth infestation cost about US$300 million dollars annually while the cost of treatment via screening programs would likely be US$2 billion annually (UCNTD 2014). Since several decades, the use of commercial anthelmintic such as albendazole, mebendazole, benzimidazole, etc. is the most common practice of controlling helminthiasis. However, there is a growing report of drug resistance in helminth parasites leading to ineffective controlling of helminthiasis through drug administration (Mphahlele et al. 2019). Plants are known for its rich phytochemicals and medicinal values and are therefore investigated for its anthelmintic properties throughout the world as an alternative to the existing commercial drugs. Several medicinal plants around the world are screened for their anthelmintic property by many researchers (Roy and Swargiary 2009; Carvalho et al. 2012; Tandon and Das 2018).

Assam is one of the north eastern states of India rich with flora and fauna. People living in this part of India, especially ethnic tribal groups perform several traditional practices to control common ailments including helminth infestation. Several plants are documented by many authors from this part of India (Swargiary et al. 2017; Panda et al. 2018; Daimari et al. 2019). In our earlier studies we have reported

several medicinal plants traditionally practiced as antidiabetic and anthelmintic agents by the tribal communities of Kokrajhar, Chirang, Baksa, and Udalguri district of Assam (Swargiary et al. 2016; Swargiary et al. 2019a, b; Swargiary et al. 2020). Traditional herbal preparations are commonly and effectively used by the tribal communities of Assam. Though a large number of plants have rich traditional uses very few have been investigated scientifically. Therefore, it is very crucial to explore medicinal values of the plants scientifically and to develop better and safer drugs. The present study attempts to investigate the phytochemistry and anthelminthic activity of plants traditionally used as deworming agents by the tribal community of Kokrajhar district of Assam, India.

Materials And Methods

Collection and Identification plants

Four medicinal plants used by the traditional healers of Kokrajhar - *Alstonia scholaris* (L.) R. Br. (BUBH2018040), *Cardiospermum halicacabum* L. (BUBH2018001), *Hydrocotyle sibthorpioides* Lam. (BUBH2018019), and *Hypericum japonicum* Thunb. (BUBH0000129) were collected from Kokrajhar locality. Herbarium sheets were prepared and the sample plant submitted to the Department of Botany, Bodoland University for taxonomic identification.

Preparation of crude plant extracts and solvent fractions

Bark of *A. scholaris*, and leaves of *C. halicacabum, H. sibthorpioides,* and *H. japonicum* were washed properly to remove dart particles and processed for methanolic crude extraction following the method described in our earlier publication (Swargiary et al. 2016). Briefly, plant parts were dried completely in hot-air oven below 50°C. Dried plants were powdered using mixture grinder. Plant powder was mixed in 80% methanol (1:5, w/v) and kept for 72 h. After that the solutions were filtered with Whatman filter paper No. 1 and the filtrate obtained is dried in a rotary evaporator. The solid material felt is collected as crude extract and kept in -20°C till further use.

Phytochemical and Antioxidant Study

Total phenolic content (TPC)

The total phenolic was estimated using Folin-Ciocalteu reagent (Iloki-Assanga et al. 2013). TPC was calculated from a calibration curve of gallic acid and results expressed as µg gallic acid equivalent (µgGAE)/mg plant extract.

Total flavonoid content (TFC)

The flavonoid content was determined following the method of Ordonez et al. (2006). TFC was calculated from the standard curve of quercetin and the values were expressed as microgram quercetin equivalent (µgQE)/mg plant extract.

Antioxidant Study

Total antioxidant capacity (TAC) assay

TAC of the plant extract was done by phosphomolybdate method using ammonium molybdate reagent (Huda-Faujan et al. 2009). TAC was expressed as µgAAE/mg plant extract.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed following the method of Iloki-Assanga et al. (2015). The FRAP activity is compared with the standard ascorbic acid and values were expressed as µg Fe^{2+} equivalent (µgFE)/mg plant extract.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH scavenging activity of methanolic plant extracts was estimated using DPPH as described by Mamta et al. (2015).

Lipid peroxidation scavenging activity (Thiobarbituric acid reactive species) assay

Lipid peroxidation inhibitory activity was studied following the modified thiobarbituric acid reactive species (TBARS) assay to measure the lipid peroxide formation using egg yolk homogenates as lipid-rich media (Okhawa et al. 1979). The coloration of the assay mixture was measured at 532 nm.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) Assay

The ABTS activity was measured following the method of Re et al. (1999) using gallic acid as standard reference.

In-vitro anthelmintic bioassay

Live, adult trematode parasite, *Paramphistomum* sp. were collected from the rumen of cow from Kokrajhar town. In-vitro anthelmintic study was carried out following Belemlilga et al. (2016). Parasites collected in phosphate buffered saline (1xPBS, pH 7.4) were brought to the laboratory and allowed to acclimatize for 30 min at 37±1ºC. Next, the parasites were incubated in a series of extract concentrations. After 24 h of treatment mortality was noted and the lethal concentration at 50% mortality (LC_{50}) was calculated. Albendazole (ALB), a broad-spectrum anthelmintic drug was used as reference chemical. Control flukes were incubated in PBS alone. For each set of experiment three replicates (n = 3) were carried out.

Cell proliferation and apoptosis study

The antiproliferative and apoptosis-inducing activity of plant extracts was studied using Dalton's lymphoma (DL) cell line as described in our earlier publication (Swargiary et al. 2021). Cell proliferation

was measured by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983; Verma et al. 2013). In brief, the cells were treated with different doses (10-200 mg/ml) of plant extracts for 24 h in a 96-well plate. Next, 10 µl of MTT reagent was added and incubated for 4 h at 5% CO_2 and 95% air at 37ºC followed by addition of 100 µl DMSO. The colour developed was measured at 570 nm using Elisa Microplate Reader. Furthermore, for apoptosis study, both the control and plant extract treated cells were stained with acridine orange/ethidium bromide (AO/Eb) for 5 min in dark cold room (Squier and Cohen 2001). The cells were then thoroughly examined for three replicates under fluorescence microscope and photographed. About 1000 cells were counted, and the percentage of apoptotic nucleus was determined based on differential staining pattern (red/green) of the nucleus.

Statistical analysis

All the statistical calculations were carried out in excel. LC_{50} and IC_{50} were calculated using OriginPro and SPSS software. All the experiments were carried out in triplicates (n = 3) and the results were represented as mean ± standard deviation (SD).

Results

Plant extracts and phytochemical content

Plants are rich in phytochemicals and contain various primary and secondary metabolites. The medicinal properties of a plant are attributed to its secondary metabolites – phenolics, flavonoid, etc. The TPC, TFC, FRAP and total antioxidant property of the plants are shown in Fig. 1. *A scholaris* showed highest TPC (86.37±7.29 µgGAE/mg extract) followed by *H. japonicum*, *C. halicacabum*, and *H. sibthorpioides*. The values of TFC ranged from 5.84±0.95 to 83.76±4.27 µgQE/mg. In all the TFC, FRAP and TAC *H. japonicum* showed strongest activity followed by *H. sibthorpioides*. The values were found to be 83.76±4.27 µgQE/mg, 287.58±8.33 µgFE/mg, and 115.65±7.34 µgAAE/mg extract, respectively for TFC, FRAP and TAC in *H. japonicum*. Values were found to be significantly different compared to other three plants at $P \leq 0.05$ level. *A. scholaris* and *C. halicacabum* showed almost similar activities in TFC, TAC and FRAP activities. The free radical scavenging activities of all the four plants is shown in Fig. 2. Antioxidant study showed a concentration-dependent inhibition of all the plants. All the plants Similar to phenolic and flavonoid content, strongest free-radical scavenging activity was observed in *H. japonicum* extract. *H. sibthorpioides* also showed strong free radical scavenging activity followed by *A. scholaris.* The IC_{50} values ranged from 28.45±1.70 µg (*H. japonicum*) to 4.27±0.39 mg (*C. halicacabum*) for DPPH scavenging property. The IC_{50} values of *H. sibthorpioides* and *A. scholaris* was found to be 231.34±17.30 and 349.64±14.10 µg. For ABTS assay, *H. japonicum* showed the strongest activity with IC_{50} value 9.97±1.77 µg followed by *H. sibthorpioides* (61.32±2.22 µg), *A. scholaris* (118.28±6.16 µg), and *C. halicacabum* (287.79±9.77 µg). All the four plants showed stronger ABTS free radical scavenging activity. Similar to DPPH, and ABTS assay, *H. japonicum* showed strongest lipid peroxidation inhibition property (IC_{50}, 57.10±3.06 µg) followed by *H. sibthorpioides* (148.13±1.0 µg), *A. scholaris* (169.22±6.88 µg), and weakest in *C. halicacabum* with IC_{50} value 372.79±19.62 µg. The reference chemical showed strongest

DPPH, ABTS, and TBARS activity with IC_{50} values 2.27±0.11 µg, 1.15±0.01 µg, and 24.33±1.13 µg, respectively.

Antiproliferative and apoptosis study

The antiproliferative and apoptosis inducing properties of all the four plants are presented in Fig. 3. The study revealed a dose-dependent cytotoxicity effects of the plants against DL cell. Of the four plants, *H. sibthorpioides* showed strongest cytotoxic property. MTT assay showed that the % death of DL cells ranged from 23±20% to 58.67±4.73%, 17.33±1.53 to 43±4.36%, 13±2.64 to 27.67±3.05% and 3±1 to 15±3.61% for *H. sibthorpioides*, *H. japonicum, A. scholaris*, and *C. halicacabum*, respectively at the concentration range of 25 – 200 mg/mL after 24 h treatment. Similar results were seen in the apoptosis study indicating the potency of plant extracts to induce apoptosis and cell death. At 200 mg/mL concentration of plant extract, the cell death was found to be 42.33±3.21%, 32.37±2.52%, 22±4% and 9.0±1.0% for *H. sibthorpioides*, *H. japonicum, A. scholaris*, and *C. halicacabum*, respectively. The study showed a positive and significant correlation ($P \leq 0.05$) between antiproliferative and apoptotic properties of the plants. Changes in the morphological features with apoptotic characteristics with red/orange nuclei, membrane blebbing, chromatin condensation, and formation of apoptotic bodies have been observed in DL cells treated with plant extracts and reference drug, cisplatin (Fig. 4). Control cells showed green nuclei with intact membrane. AO/Eb staining showed higher density of apoptotic cells in *H. sibthorpioides* treatment followed by *H. japonicum,* and *A. scholaris*. *C. halicacabum* extract showed the lowest apoptosis-inducing property cells.

Anthelmintic activity

The trematode parasite, *Paramphistomum* sp. when exposed to different concentrations of plant extract showed dose-dependent mortality after 24 h treatment. Fig. 5 showed the percent mortality of the parasite treated with different concentrations of extracts. *H. japonicum* showed the strongest parasite mortality followed by *H. sibthorpioides, C. halicacabum*, and *A. scholaris*. The plant also showed better activity compared to reference drug, albendazole. At highest dose of 2 mg/mL, *H. japonicum* showed almost 100% mortality in helminth parasites. Similarly, at 20 mg/mL plant of *H. sibthorpioides* and *A. scholaris*, helminth parasites showed almost 100% and 60% mortality. While, *C. halicacabum* attained almost 100% mortality at 40 mg/mL. The LC_{50} value was found to be 212.87 µg/mL, 5.36 mg/mL, 13.40 mg/mL, and 18.40 mg/mL for *H. japonicum, H. sibthorpioides, C. halicacabum*, and *A. scholaris*, respectively. Similarly, the LC_{50} value of reference drug, albendazole was found to be 3.69 mg/mL. The control, untreated parasite lived up to ~73 h.

Discussion

Free radicals are by-products of biological reactions which produce several health effects. Biomolecules, called antioxidants, can neutralise and scavenge those free radicals. Plants are known to contain strong antioxidant property due to its rich secondary metabolites. Polyphenolics and flavonoids are among the

most important phytochemicals with rich antioxidant property. Phytocompounds can provides health benefits in many ways such as substrates for biochemical reactions, co-factors of enzymatic reactions, enzyme inhibitors or stimulators, scavengers of reactive or toxic chemicals, and many more (Dillard and German 2000). Plants, in addition to primary metabolites, also produce secondary metabolites that help in normal growth, development, and defence system of plants. Among all the different type of secondary metabolites, phenolics are the most important because of its promising antioxidant properties (Horwitt 1991). The antioxidant property of plants may be attributed to its innate ability to synthesize non-enzymatic antioxidants such as ascorbic acid, glutathione as well as secondary metabolites such as phenolic compounds. The present study revealed considerable amount of phenolics and flavonoid content in all the four plants. Many findings suggest that the high TPC and TFC can improve biochemical indices of oxidative damage (Serafini et al. 1996; Stein et al. 1999). The continuous generation of free radicals may cause severe complication if in excess. To minimise such complications, intake of antioxidant is always beneficial. In the present study, *H. japonicum,* and *H. sibthorpioides* showed higher phenolic and flavonoid contents among the four plants. Similar kind of study showed comparable data of phenolics and flavonoid content (Irshad et al. 2012). The methanolic peel extract of *Citrus grandis* also showed comparable data with our findings (Dibya et al. 2016). Like most of the other studies, we also found potent antioxidant property in all the plants. Statistical analysis revealed that the in vitro antioxidant study by TAC, FRAP, DPPH, ABTS and TBARS assays showed positive correlation to TPC and TFC which reinstate the function of phenolics as potent antioxidant molecule.

Growth and development of an organism depends on the proper regulation of cell division and death. Any deviations from the normal physiology of cell growth, division, and death leads to the development of disease and complications. Unregulated cell division and growth is an important characteristics of cancer disease (Wong 2011). Apoptosis is a cellular mechanism that causes normal cell death, also known as programmed cell death. Cancer cells are known to avoid apoptosis and normal cell death cascade leading to uncontrolled cell division. Because of its cellular importance, apoptosis-inducing drugs have been shown to be the centre of new anti-cancer therapy (Elmore 2007). Several studies have been carried out to explore the antiproliferative and apoptosis-inducing properties of several medicinal plants (Rais et al. 2019; Khurshid et al. 2020). Phytochemicals and bioactive compounds isolated from plants were also investigated for anti-cancer activity in numerous cell lines (Kamaruddin et al. 2019; Erdogan et al. 2020). In the present study, *H. sibthorpioides*, and *H. japonicum* showed better antiproliferative and apoptosis-inducing property compared to other plants. Both the plants showed high cell mortality (~50%) at the highest dose of plant extract. The various phytocompounds present in the plant extract and their synergistic effects may have contributed to the antiproliferative and apoptotic activity. It is reported from many studies that studies that the crude extracts of plants show lesser biological activity compared to the isolated compounds (Lowe et al. 2013; Alasmary et al. 2018; Swargiary et al. 2021). Cisplatin, the reference chemical showed a significant difference (at $P \leq 0.05$ level) in both antiproliferative and apoptotic inducing capacity compared to medicinal plants.

Helminth infestation is one of the 17 Neglected Tropical Diseases of the world (WHO 2010). According to recent publication India stand global leader in many of the NTDs including helminthiasis and other vector

borne diseases (Hotez et al. 2018). Most of the time development of drug resistance by helminth parasites is recognised as the major hurdle in effective controlling of helminthiasis. As an alternative or supplementary to the existing system of synthetic medicines plants are regarded as an effective tool in dealing helminthiasis. In the present study, we have investigated the anthelmintic property of four medicinal plants consumed by the tribal groups of Bodoland Region of Assam. In a similar study, Kumar et al. (2013) established strong anthelmintic property of the bark extract of *A. scholaris*. Leaves of *H. japonicum* showed the strongest anthelmintic activity among all the plants while *A. scholaris* showed the weakest activity. *H. japonicum* is also known to contain several pharmacological properties such as anticancer, hepatoprotective, antiviral, and immune-boosting activity (Liu et al. 2014). Several other studies have reported the anthelmintic activity of many plants (Roy and Swargiary 2009; Irshad et al. 2010; Swargiary et al. 2017). Wahyuni et al. (2019) also reported *in vivo* anthelmintic activity of four plants belonging to *Cassia* sp. from Indonesia showing highest activity in *Cassia surattensis*. Citrus is an important fruit plant having tremendous medicinal property. Several species of citrus have been investigated for its therapeutic potential by many researchers. Accordingly, *C. sinensis, C. medica* and *C. reticulata* have been reported to possess anthelmintic property against *Haemonchus contortus* and *P. posthuma* (Gainza et al. 2015; Aryal et al. 2017). Studies have revealed positive relationship between the phenolics and pharmacological properties of the plants (Akkari et al. 2016). The phenolic compounds of plants can interfere with the oxidative phosphorylation pathway of helminth parasites leading to the inhibition of ATP synthesis and induce mortality (Athnasiadou et al. 2001). Studies also reported that the phenolic compounds can bind to glycoprotein on the cuticle of helminth parasites and causes death (Salhan et al. 2011). The present study also observed positive correlation between the antioxidant, antiproliferative, and anthelmintic activity of the plants. The present study observed positive correlation between antioxidant property, antiproliferative, and anthelmintic activity of the plants. The secondary metabolites responsible of high antioxidant property may also have contributed to strong antiproliferative and anthelmintic property of the plants in the present study. The cytotoxicity property of the plants is directly correlated to the mortality of cells. Higher the cytotoxicity property of plants higher cell death. The present study also revealed that higher the cytotoxicity of the plant stronger the anthelmintic activity.

Conclusions

The current study validates the traditional knowledge and faith of people practicing since ancient times. All the four plants showed promising antiproliferative and anthelmintic activity. The rich secondary metabolites present in the plant extract may have synergistic effect in contributing the cytotoxicity and anthelmintic property of the plants. *H. sibthorpioides* and *H. japonicum* showed promising antiproliferative and anthelmintic property. However, further phytochemical characterisation and in vivo bioassay need to be carried out to explore the bio-active compounds responsible for antiproliferative and anthelmintic activity of the plants.

Declarations

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Availability of data and material: The authors confirm that the data supporting the findings of the study are available within the manuscript.

Author Contributions: AS involved in designing the study, statistical calculations and writing of the manuscript, MKR carried out the antioxidant and anthelmintic study, and AKV carried out the antiproliferative and apoptosis study. All authors read and reviewed the final manuscript.

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Figures

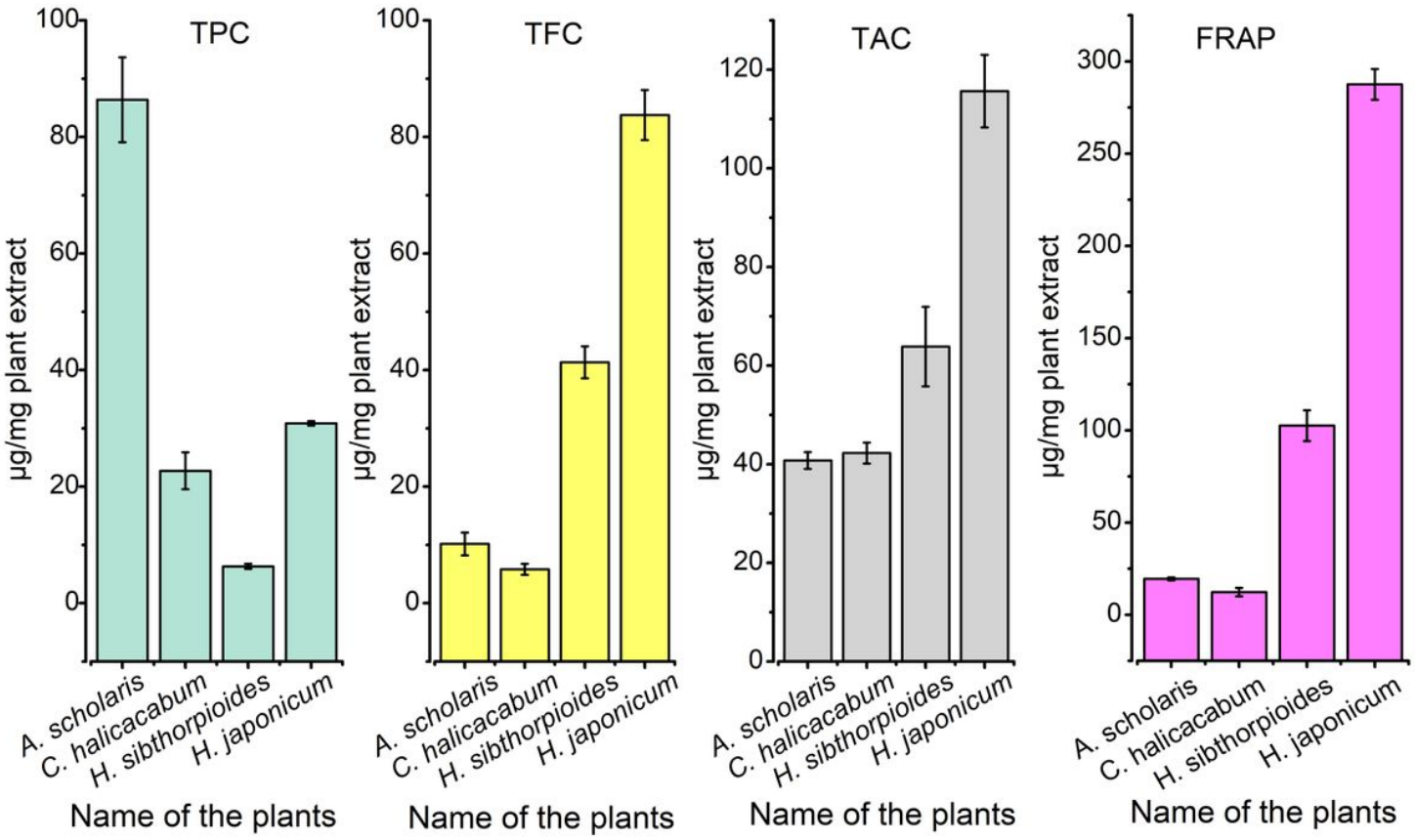

Figure 1

Total phenolic (TPC), flavonoid (TFC), total antioxidant (TAC), and ferric reducing (FRAP) capacity of the plants. Values are expressed as mean ± SD, n = 3

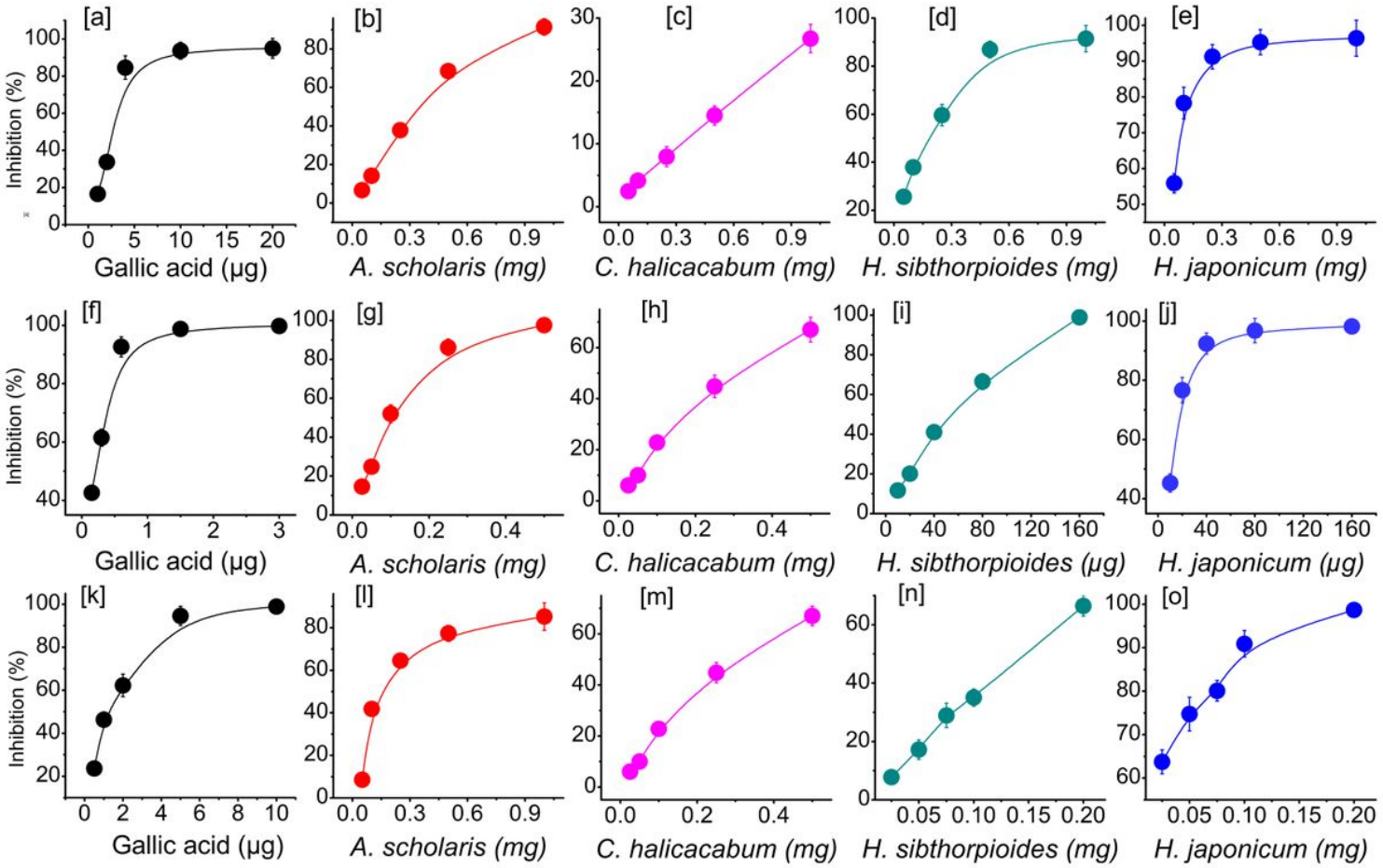

Figure 2

Antioxidant activity of the plants (a-e) DPPH, (f-j) ABTS and (k-o) TBARS activity. Values are expressed as mean ± SD, n = 3

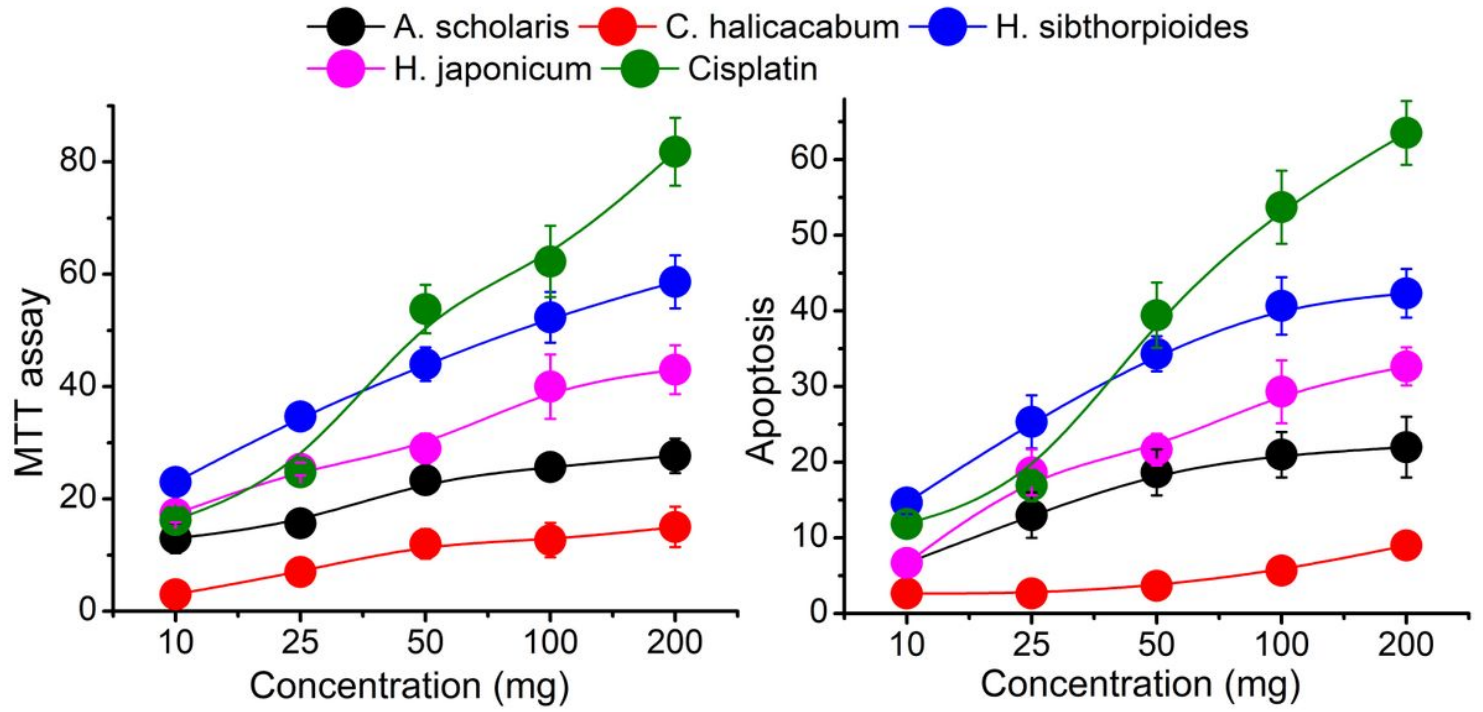

Figure 3

Antiproliferative and apoptosis-inducing activity of the plants. Values are expressed as mean ± SD, n = 3. Mortality values are significantly different between Cisplatin and plant extract treatment at P≤0.05

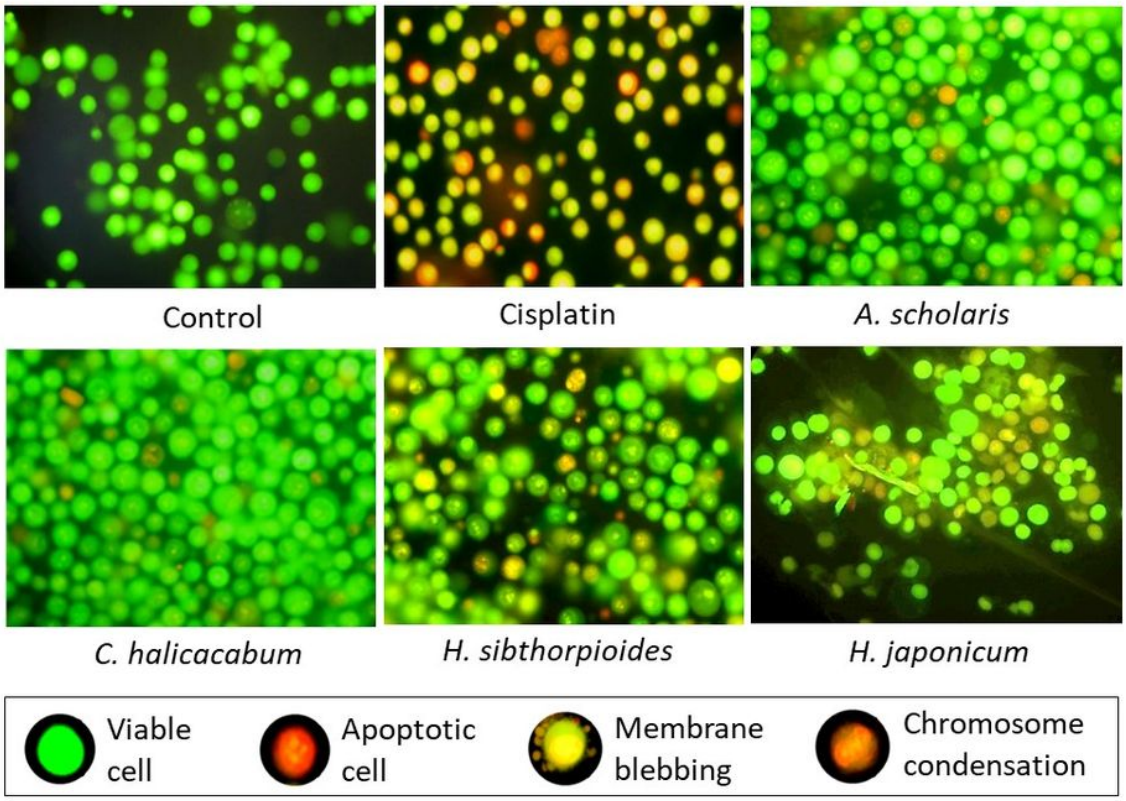

Figure 4

Apoptotic features of plant extract-treated cells observed under fluorescence microscope after AO/Eb staining. Cisplatin is used as reference drug. Apoptotic cells are shown in red/orange nucleus, control cells showed green nucleus

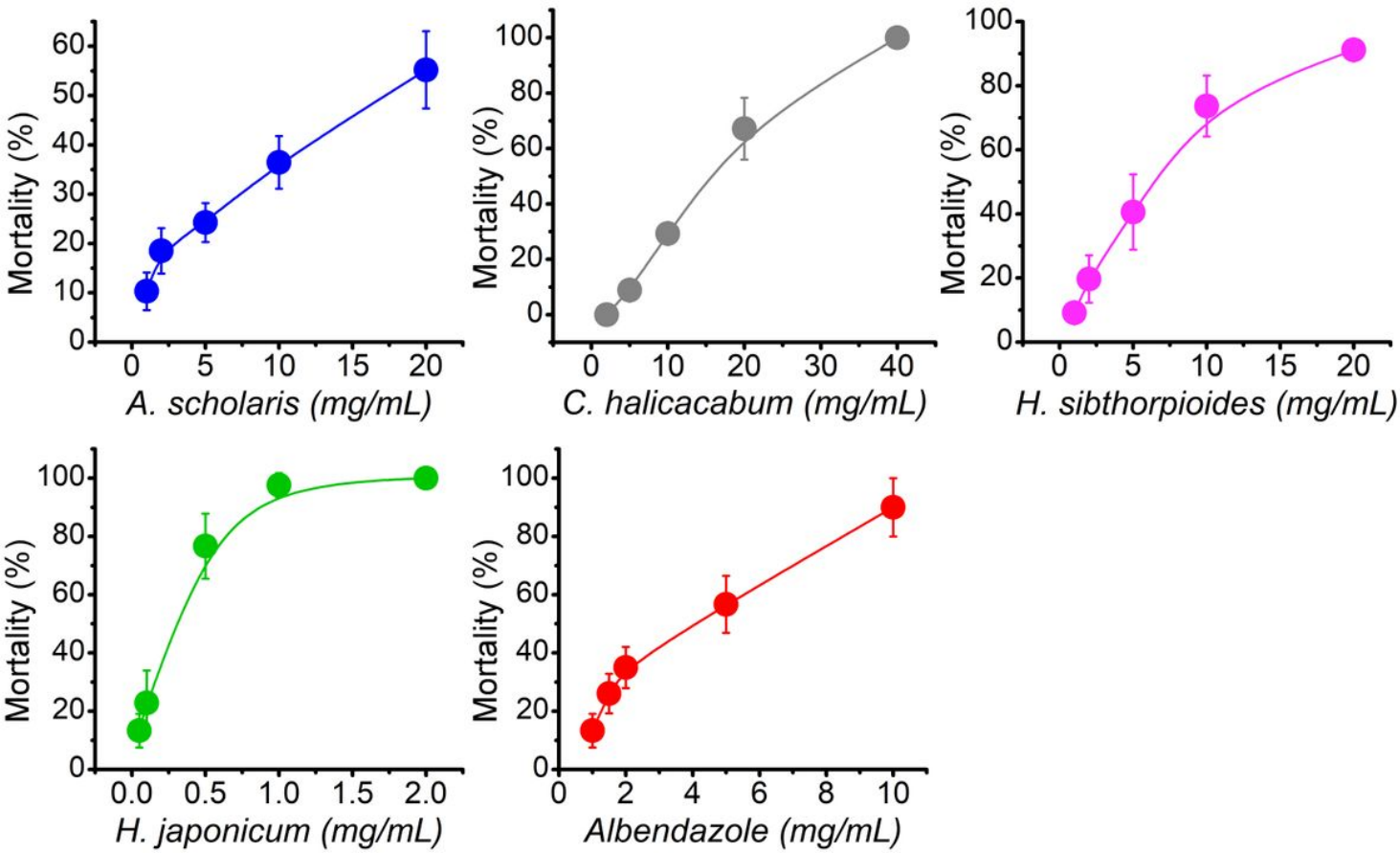

Figure 5

Mortality of helminth parasites at different test concentrations of plant extracts. Values are represented as mean ± SD, n = 3