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Evaluation of the antioxidant activity of novel south Indian plant *Commelina forskaolii* and its prospective anticancer activity in Hep G2 cells

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Oxidative stress plays a part in the progression of cancer and various types of diseases by damaging the DNA. Generally, plants have high antioxidant potency so that they can reduce oxidative stress. Plants are found to be effective in treating various types of cancers because of their antioxidant property. *Commelina forskaolii* was used in the primitive ages to treat various diseases. This study evaluated the antioxidant and cytotoxic potential of the novel medicinal plant *C. forskaolii* followed by qualitative and quantitative phytochemical analysis. DPPH, ABTS and hydroxyl radical scavenging activity were used to determine the antioxidant potential. The HepG2 cell line was used to study anticancer activity because it is a widely used cell line to study hepatotoxicity and hepatocellular carcinoma. The plant showed favourable antioxidant activity by scavenging various radicals such as DPPH, ABTS and hydroxyl radicals due to the presence of phytochemicals such as alkaloids, flavonoids, glycosides, tannins and terpenoids. Compared to the other plant extracts, the aqueous extract was found to be more potent in scavenging free radicals. The plant showed cytotoxicity against the HepG2 cell lines. The medicinal plant *C. forskaolii* contains a variety of phytochemicals and has high antioxidant potential. The plant has a capacity to inhibit the growth of liver cancer cells.

Keywords: Anticancer, Antioxidant, *Commelina forskaolii*, Oxidative stress, Phytochemicals, Sequential extraction

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Introduction

Oxidative stress, which is brought on by an imbalance between free radical creation and neutralization, plays a role in the development of cancer and a variety of diseases. Oxidative stress is produced by free radicals such as hydroxyl (OH^-), hydroperoxide (H_2O_2), and superoxide (O_2^-) radicals that, in healthy human cells, are coupled with biological macromolecules including proteins, lipids, and DNA to become stable. Additionally, these radicals cause DNA, protein damage, and lipid peroxidation. Damage brought on by oxidative stress has been related to a severe illness like cancer[1]. Traditionally, plants are consumed as medicine in various countries because of the bioactive components that occur in various plants as secondary metabolites grouped as phytochemicals with biotic roles. Plant herbal polyphenols are used as probable antioxidants for human well-being[2].

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The evaluation of plant materials often involves assessing their antioxidant capabilities. The characteristics such as free radical inhibition, oxygen scavenging, peroxide decomposition, and metal inactivation represent some of the mechanisms associated with antioxidant activity[3]. Human studies have found a link between eating whole fruits and vegetables and preventing diseases like coronary artery disease, cancer, metabolic disorders, and joint problems, as well as improving heart health and neurological health, decreasing the incidence of cancer, extending life expectancy, and reducing overall death rates[4]. Currently, medicinal plant research is increasingly becoming important as an alternative approach to cancer therapeutics[5]. A medicinal plant from the genus Commelina called *Commelina forskaolii* Vahl was used for this investigation. It is a perennial, fast-growing herbaceous shrub with the common name "rat's ear" indigenous to Africa, Arabia, and India. The *Commelina diffusa*, found in this genus, was used by traditional healers to treat cuts, burns, and joint

discomfort. It has been used to treat urinary tract infections, respiratory infections, diarrhoea, and haemorrhoids throughout Africa and southern Asia. The species Commelina is a perennial or biennial shrub in the family Commelinaceae. There are about 170 different types in northern and tropical areas. Numerous Commelina extracts have been proven to stop the growth of various cancer cell types in recent investigations[6]. The alcohol-based extract of *Commelina benghalensis* inhibited the proliferation of leukemic Jurkat-T cells[7]. The prevalence of liver cancer is expanding globally, and it remains to be problematic for global well-being. Over 1.0 million people each year are predicted to be impacted by liver cancer by 2025[8]. The most frequent histological type of liver cancer (>80%) is hepatocellular carcinoma. Globally, the geographic distribution of liver cancer varies greatly. In contrast to Asia, notably East and South Eastern Asia, where hepatitis B virus infection is common, North and South America have comparatively low incidences of the disease[9].

In the current study, the plant *C. forskaolii* was qualitatively and quantitatively analysed for phytochemicals using sequential extracts (low polar to high polar solvent). The extracts having high phytochemical activity were carried out for various antioxidant activities such as DPPH, ABTS, and hydroxyl radical scavenging activity. Plant extract with high antioxidant potential was used to study the anticancer activity using HepG2 cells because it was widely used in pharmacotoxicological research and drug metabolism.

Materials and Methods

Materials

All chemicals were purchased from Hi Media, and Sigma Aldrich was used in the study. The cell lines were purchased from the National Centre for Cell Science Pune Maharashtra.

Plant collection and identification

The Plant was collected from the Western Ghats of Coimbatore and identified by the Botanical Survey of India, Coimbatore (Certification Number: BSI/SRC/5/23/2022/Tech/361). The plant materials were rinsed twice under tap water to remove the mud and filth. The roots of the plant were removed, and the aerial part of the plant was used in the study. Plants were chopped into little small and dried in shadow at 35°C (12-hour /12-hour light and dark cycle) at 35°C. Plant materials that had been fully dried were ground into a powder using a mechanical blender. For the soxhlet extraction, different polar solvents, including petroleum ether (PE), chloroform (CF), ethyl acetate (EA), methyl alcohol (MET), and water (WA), have been used.

Extraction of plants using the Soxhlet Method

Using the Soxhlet extraction method, 10g of plant powder was extracted overnight using the appropriate solvent temperatures in 250 mL of each of the following solvents: petroleum ether, chloroform, ethyl acetate, methyl alcohol, and water. The resulting extracts were placed in a vacuum evaporator to remove excess solvents. The dried extract was then weighed for yields and used for further experiments[10].

Qualitative phytochemical analysis

Phytochemical investigation of sequential extracts was performed using the methods described in Mujeeb *et al.*[11] with slight modifications.

Quantitative phytochemical analysis

Estimation of alkaloids

1 mL of the test extract was added with 5.0 mL of pH 4.7 phosphate buffer, 5.0 mL of bromocresol - green reagent, and 4.0 mL of chloroform. Next, the mixture was shaken. The extracts were combined in a 10-mL volumetric flask, and the volume was adjusted by diluting with chloroform. The mixture formed in chloroform was evaluated for absorbance at 470 nm compared to a blank produced in the same way but without extract. To compare the assay findings to atropine equivalents, atropine was used as a reference material[12].

Estimation of flavonoids

The total flavonoid was quantified using the aluminium chloride method followed by (Saeed *et al.*)[13] using catechin as a reference. 4.0 mL of water and 1.0 mL of the plant extract were added to a 10 mL volumetric flask. After five minutes of waiting, 0.3 mL of 10% aluminium chloride and 5% sodium nitrite were added. After 6 minutes of incubation at 37°C, 2.0 mL of 1 M sodium hydroxide solution was added to the mixture. The resulting volume was immediately diluted with 10 mL of deionized water. The absorbance of the mixture at 510 nm was measured spectrophotometrically using a blank solution. The outcomes were displayed as catechin equivalents[14].

Estimation of total tannin

The total tannin was quantified using the method described by (Kulkarni *et al.*)[15]. After adding 20% sodium carbonate solution and deionized water, 1.0 mL of plant extract was combined with 0.500 mL of folic-Ciocalteau's reagent. Thirty minutes at room temperature were given for the reaction mixture to stand. Using a UV-visible spectrophotometer, the optical density at 725 nm was obtained after the supernatant was produced by centrifugation at 6,000 rpm. The absorbance of various standard tannic acid concentrations was shown for a standard graph using various concentrations of prepared standard tannic acid. Tannic acid equivalent (mg/g) was used to measure the amount of tannin in sample[14].

Estimation of cardiac glycosides

5 mL of plant extract and 10 mL of freshly made Baljet's reagent (95 mL of 1% picric acid solution and 5 mL of 10% sodium hydroxide were added to determine the presence of cardiac glycosides. After an hour, the mixture was diluted with 20 mL of distilled water, and a UV/VIS spectrophotometer was used to detect the absorbance at 495 nm. For the standard curve, Digoxin was prepared in 10 mL of different concentrations (ranging from 12.500 to 100.00 mg/L). Digoxin (mg/g) of plant extracts expressed the total glycosides from three replicates[16].

***In vitro* antioxidant investigation**

DPPH assay

DPPH assay was analysed by the method used by Muthoni *et al.*[17] with some variations. The MET (Methanolic extract) and WA (Water extract) plant extracts were used with five concentrations (20, 40, 60, 80, and 100 µg). The same was repeated with ascorbic acid, which was taken as standard. 1.0 mL of several concentrations were taken in separate test tubes. 0.5 mL of 0.3 mM DPPH was added in all tubes. This was repeated in the standard tubes. The solution was mixed well and allowed for 15 minutes in the dark. This procedure was repeated thrice. The optical density was observed at 517 nm using a spectrophotometer. The percentage of inhibition was calculated by the following formula,

$$\text{DPPH Scavenged (\%)} = \frac{\text{Abs}_1 - \text{Abs}_2}{\text{Abs}_1} \text{X } 100$$

where, Abs_1 - Absorbance of control and Abs_2 - Absorbance of sample

The inhibitory concentration IC_{50} was also calculated.

ABTS assay

The ABTS assay was performed using the method followed by Bano *et al.*[18]. ABTS was prepared by dissolving 7 mM ABTS in a pH 7.4 buffer and adding 2.45 mM potassium persulfate. The solution was kept in the dark for 12 to 16 hours at room temperature. Increasing concentrations of the plant extract were compared with a Trolox as a standard[19], mixed with ABTS, and incubated in the dark for 30 min at 37°C temperature. The percentage of inhibition at the absorbance at 414 nm was calculated by the following formula,

$$\text{ABTS Scavenged (\%)} = \frac{\text{Abs}_1 - \text{Abs}_2}{\text{Abs}_1} \text{X } 100$$

where, Abs_1 - Absorbance of control and Abs_2 - Absorbance of sample

The inhibitory concentration IC_{50} was also calculated.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was done using the method used by Pavithra *et al.*[20]. The MET and WA plant extracts with five several concentrations (20, 40, 60, 80, and 100 µg) were used, and positive control, mannitol (20, 40, 60, 80, 100 µg), a traditional hydroxyl scavenger[21], were taken, and 1 mL of iron EDTA solution and 0.5 mL of EDTA solution, and 1 mL DMSO and 0.5 mL of Ascorbic acid is added to it. The mixture was boiled for 15 minutes at 80-90°C. After incubation, 1 mL of ice-cold TCA and 3 mL of Nash reagent were added and incubated at room temperature for 15 minutes. The absorbance was read at 412 nm. The inhibition percentage was obtained by associating the sample with the standard.

$$\text{(\%) of radical scavenged} = \frac{\text{Abs}_1 - \text{Abs}_2}{\text{Abs}_1} \text{X } 100$$

where, Abs_1 - Absorbance of control and Abs_2 - Absorbance of sample

The inhibitory concentration IC_{50} was also calculated.

***In vitro* anticancer activity**

The MTT assay was used to measure the cancer activity of substances on HepG2 cells. The frequently used method depends on tetrazolium-based dyes like the MTT and WST assays. This approach exploits the capability of viable cells to convert a tetrazolium salt into formazan crystals[22]. This cell line was grown from liver biopsies taken from a 15-year-old Caucasian male with differentiated hepatocellular

carcinoma[23,24]. In 96-well plates, cells (1×10^4 /well) were plated in 0.1 mL of medium per well. Seventy-two hours should be spent incubating at 5% CO_2. The samples were then put in varied quantities in 0.1% DMSO solution for 24 hours in a CO_2 incubator at 5% CO_2. Photographs were taken after viewing the images with an inverted microscope at 40X. MTT reagent was added after the media was removed, 20 µL/well, incubated for 4 hours in the dark, and 1 mL of DMSO was added following the incubation. The absorbance at 540 nm was used to recognize viable cells. The 50% inhibition of cell viability (IC_{50}) value was determined.

$$\% \text{ cell viability } = \frac{A_{540} \text{ of plant extract treated cells}}{A_{540} \text{ of control cells}} X\ 100\%$$

Statistical Analysis

GRAPHPAD PRISM 9 was used for statistical analysis. The average SD of the triplicates was used to reflect the outcomes of each experiment. One-way ANOVA (Dunnett's test) was used to examine variance and significant variations between the means with P <0.05. Probability level P <0.0001 (****) and P = 0.01 to 0.05(*).

Results and Discussion

Qualitative phytochemical analysis

Table 1 illustrates the presence of phytochemicals in various extracts of *Commelina forskaolii.* Compared to the other organic solvents, a high yield in g percentage was obtained in the aqueous extract. From the preliminary phytochemical investigation, the phytochemicals present in the *Commelina forskaolii* were alkaloids, flavonoids, glycosides, terpenoids and tannins. Compounds derived from plants as secondary metabolites exhibit enhanced effectiveness and serve as efficient drugs for various diseases[25]. Alkaloids have a wide range of therapeutic prospects in today's medicine due to their powerful and diverse actions, which include analgesic, anti-hyperglycaemic, anticancer, antiarrhythmic, and antibacterial properties. Other alkaloids have both psychotropic and stimulating effects on the central nervous system. Despite a wide range of uses in history, only a few alkaloids are actively marketed as medicines[26]. Additionally, flavonoids have a significant influence on the development of cancer and anti-inflammatory capabilities. Inactivating carcinogens, triggering apoptosis, causing an arrest of the cell cycle, and blocking angiogenesis are some ways that these plant extracts work to exert their action. The blocking of the enzymes xanthine oxidase, 5-lipoxygenase, and cyclooxygenase-2, which are involved in the promotion and development of tumours, and the inhibition of Reactive Oxygen Species generation are two mechanisms through which flavonoids have been shown to reduce the growth of tumour cells[27]. The use of tannins with antioxidant qualities in food and medicine is widespread. To determine the pertinent antioxidant activity of tannins, numerous investigations have been done recently. Due to their antioxidant properties, which include protecting against osteoporosis, cancer, and cardiovascular disease, tannins have been receiving more attention[28]. Since aqueous and methanol extracts possess major phytochemicals, it was carried out for further analysis.

Quantitative phytochemical analysis

Table 2 shows that the aqueous extract has more phytochemicals than the methanol extract. The methanol extract exhibited a flavonoid content of

Table 1 — Qualitative phytochemical analysis of various extracts of *Commelina forskaolii*

Phytochemicals	Solvents				
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Water
Alkaloids	+	-	-	+	+
Steroids	-	-	-	+	+
Flavonoids	-	-	-	+	+
Tannins/phenols	+	-	+	-	+
Amino acids & Proteins	-	-	-	-	-
Oil & fats	-	-	-	-	-
Carbohydrate	-	+	-	-	-
Glycosides	+	-	+	+	+
Saponins	-	-	-	-	-
Terpenoids	-	-	+	-	+
% of yield (g%)	0.55	0.27	0.43	0.56	2.3

(+) Present and (-) Absent

10±0.63 mg/g catechin equivalent, while the aqueous extract showed 23±0.43 mg/g catechin equivalent. Alkaloids in the methanol extract were found to be 21±0.74 mg/g Atropine equivalent, whereas the aqueous extract showed a higher concentration of 46±0.41 mg/g Atropine equivalent. For cardiac glycosides, the methanol extract contained 8±0.46 mg/g of Digoxin equivalent, while the aqueous extract demonstrated a higher concentration at 16±0.51 mg/g of Digoxin equivalent. Additionally, the total tannin content in the aqueous extract was measured to be 44±0.39 mg/g tannic acid equivalent. Alkaloids have proven therapeutic applications in humans, ranging from pain management to treating neurological disorders. Flavonoids have the capacity to trigger signaling pathways that result in apoptosis, effectively impeding the survival and proliferation of cancer cells.

***In vitro* antioxidant investigation**

Because of their dietary and therapeutic benefits, plant-based natural antioxidants have drawn the interest of numerous researchers. It is impossible to assess the ability of medicinal plants to scavenge free radicals using a single approach alone due to the complicated reaction of phytochemicals and other naturally occurring antioxidants. Investigating natural antioxidants and identifying and isolating antioxidant compounds from medicinal plants holds significant potential in addressing redox imbalance and preventing diseases associated with free radicals[29]. Therefore, using many test techniques and assays is necessary to verify the antioxidant capability of a plant sample[30]. Therefore, it is useful to use multiple methods (DPPH, ABTS, and hydroxyl) to explain plant extracts antioxidant capabilities thoroughly. Each approach merely approximates the antioxidant capacity due to different reaction processes[31]. The most common method for evaluating the capacity of a substance to act as an antioxidant is this test. The medical community is paying close attention to plant-derived supplements and pharmaceuticals as a means of defending against ROS and ROS-mediated abnormalities[32]. The excessive production of free radicals damages cells and leads to various dysfunctions in people, including atherosclerosis, myocardial infarction, cancer, and neurodegenerative illnesses. However, natural antioxidant chemicals are helpful for treating different chronic diseases and repairing cell damage caused by free radical production[33].

Table 2 — Quantification of phytochemical using aqueous extracts of *Commelina forskaolii*

Phytochemicals	Methanol extract	Aqueous extract
Flavonoids (mg/g catechin equivalent)	10±0.63	23±0.43
Alkaloids (mg/g Atropine equivalent)	21±0.74	46±0.41
Cardiac Glycosides (mg/g of Digoxin equivalent)	8±0.46	16±0.51
Total Tannin content (mg/g tannic acid equivalent)	-	44±0.39

Mean±SD for triplicate. Values are expressed as Mean±SD for triplicate with significance of P <0.05

DPPH assay

A hydrogen radical or an electron can be accepted by the stable, diamagnetic molecule DPPH to make it more stable. Due to its unusual electron, it possesses a significant optical density at 517 nm, which disappears when the electron pairs off. The Beer-Lambert rule is followed over the applied range of absorption in 0.5 mM alcoholic solutions, which are also highly coloured[34].

Plant extracts show an intensity-dependent rise in antioxidant activity as the concentration rises (Fig. 1a).

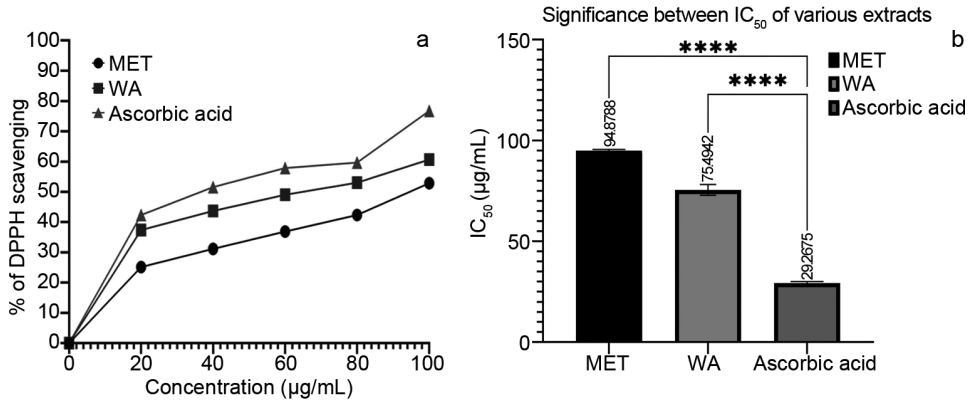

Fig. 1 — a) DPPH activity of plant extracts; and b**)** Significance between IC_{50} of MET, WA and ascorbic acid with the significance of P <0.05.

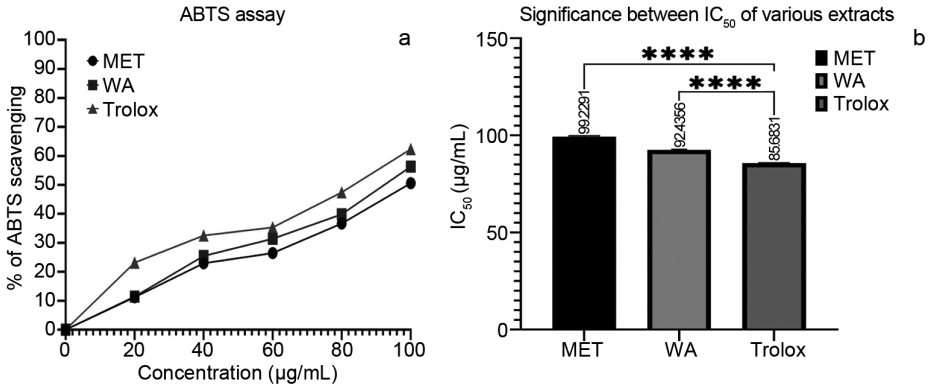

Fig. 2 — a) ABTS activity of plant extracts; and b**)** Significance between IC_{50} of MET, WA and ascorbic acid with the significance of P <0.05.

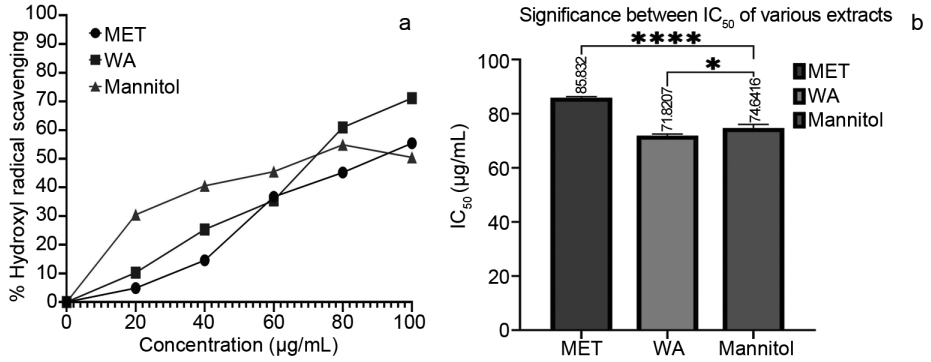

Fig. 3 — a) Hydroxyl radical scavenging activity of plant extracts; and b) Significance between IC_{50} of MET, WA and ascorbic acid with the significance of P <0.05.

The IC_{50} of MET, WA and ascorbic acid were found to be 94.87, 75.49 and 29.26 µg/mL. Both the MET and WA extracts show significant differences compared to the ascorbic acid (Fig. 1a). Comparatively, the WA extract shows more activity than the MET extract (Fig. 1b).

ABTS assay

Plant extracts show an intensity-dependent rise in antioxidant activity as the concentration rises (Fig. 2a). The IC_{50} of MET, WA, and Troloxwas found to be 99.22 µg/mL, 92.43 µg/mL and 85.68 µg/mL. Both the MET and WA extracts show significant differences compared to the Trolox (Fig. 2a). Comparatively, the WA extract shows more activity than the MET extract (Fig. 2b).

Hydroxyl radical scavenging activity

Plant extracts show an intensity-dependent rise in antioxidant activity as the concentration rises (Fig. 3a). The IC_{50} of MET, WA and mannitol was found to be 85.83, 71.82, and 74.64 µg/mL. Both the MET and WA extracts show significant differences compared to the mannitol (Fig. 3a). Comparatively, the WA extract shows more activity than the MET extract and mannitol (Fig. 3b).

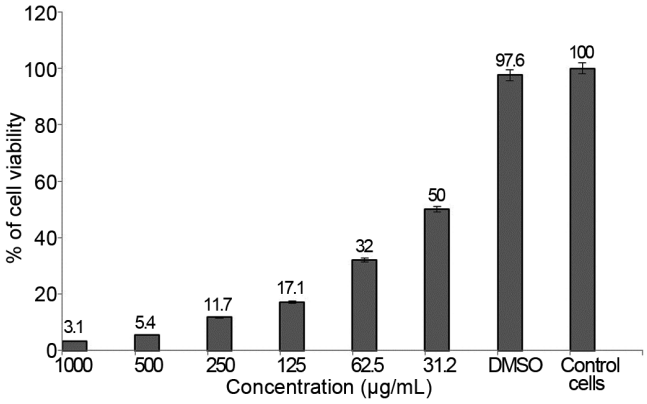

Fig. 4 — Anticancer activity of plant extract.

***In vitro* anticancer activity**

The MTT assay has been used to investigate the *in vitro* cytotoxic potential of plant extracts, and the results showed that the WA extract was effective. The outcomes also presented that the plant extract anticancer activity effect increases with concentration (Fig. 4). Plant extracts inhibit cell proliferation at

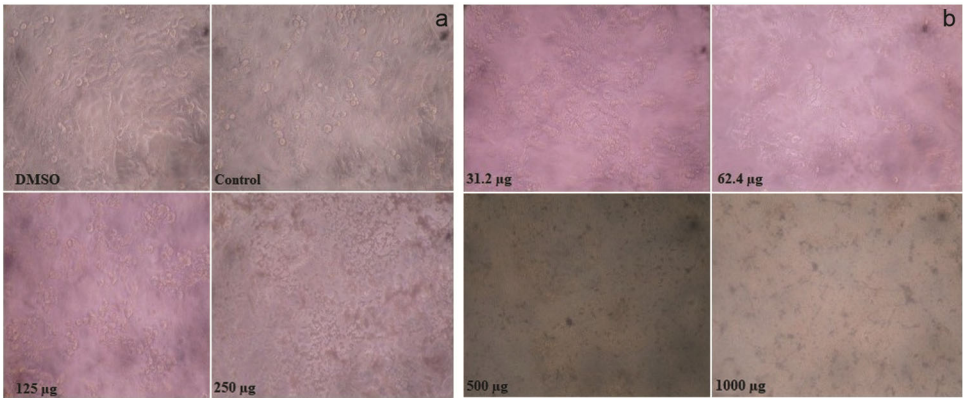

Fig. 5a-b — Inverted microscopic images of HepG2 cells at 40X treated with various concentrations.

IC_{50} 56.25 µg/mL. The morphological changes in cells with increasing concentrations of WA extract are shown in (Fig. 5a-b). Numerous anticancer drugs have been created using botanical ingredients. Malignant cell apoptosis induction has been regarded as a key treatment strategy for cancer. It has been determined that examining apoptotic activators from various plants can be a useful source for preventing cancer, both through the use of identified bioactive chemicals and crude extracts derived from the plant[35].

Conclusion

A variety of secondary metabolites, such as alkaloids, flavonoids, glycosides, tannins, and terpenoids, are found in *Commelina forskaolii.* Comparing other solvents, major phytochemicals are found to be in aqueous and methanolic extracts. Aqueous extract contains more alkaloids, flavonoids, glycosides and tannins than methanolic extract. The aqueous and methanolic extracts of plant extracts showed favourable antioxidant potential against DPPH, ABTS, and Hydroxyl radicals. Comparatively, aqueous extract showed more antioxidant activity than methanolic extract. The plant can inhibit the growth of liver cancer HepG2cells. In the future, we will isolate bioactive compounds from the plant and study the i*n vivo* anti-cancer potential of the plant extract.

Conflict of interest

There is no conflict of interest regarding the publication of this manuscript.

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