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Polyphenol content, antioxidant and antiproliferative effects of a plant mixture composed of four medicinal plants with hepatoprotective potential

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Reactive oxygen species (ROS), known as normal by-products of various cellular processes, are involved in several human diseases, including cancer and liver diseases. The present study aimed to (i) determine the polyphenol contents, (ii) assess the antioxidant, and (iii) antiproliferative effects of a mixture composed of *Balanites aegyptiaca*, *Calotropis procera*, *Cochlospermum tinctorium*, and *Acanthospermum hispidum*. These plants are reported to elicit various properties, including antioxidant, hepatoprotective, and anticancer. The phytochemicals were screened qualitatively, and Folin and Ciocalteu as well as aluminium chloride were used as reagents, to measure the total phenolics (TPC) and flavonoids (TFC), respectively. The antioxidant assay used four assays: DPPH, ABTS, FRAP, and LPO. The antiproliferative effect of the mixture was investigated on the human hepatocarcinoma cell line Hep3B. The phytochemical screening revealed the presence of saponins, flavonoids, tannins, sterols, coumarins, and triterpenes. The mixture seems richer in total phenolics (208.81 ± 1.13 mg GAE/g extract) than flavonoids (10.74 ± 1.44 mg QE/g extract). Regarding the radical scavenging activity, the mixture significantly inhibited ABTS radicals with an IC_{50} value of 11.61 ± 0.0006 µg/ml. Moreover, it was potent in reducing ferric iron (133.06 mol AAE/g) and inhibiting lipid peroxidation (inhibition percentage equal to 56.39%). The mixture showed a considerable antiproliferative effect against the Hep3B cells with an IC50 value of 1.1 ± 0.75 µg/mL The beneficial effects attributed to the mixture may be explained by the presence of phenolics known for their potent antioxidant properties. The study findings suggest a promising synergistic plant combination for protecting the liver from oxidative stress and cancer. However, more detailed studies are needed to identify and confirm the antioxidant and antiproliferative mechanisms of action.

Key words: Plant mixture, polyphenol content, hepatoprotective, antioxidant, antiproliferative.

INTRODUCTION

Worldwide, at least two million deaths are attributable to liver diseases such as cirrhosis, viral hepatitis, and liver

cancer (Devarbhavi et al., 2023). The latter is the fourth significant cause of cancer-related deaths globally (Borrello and Mann, 2022). One of the significant causes of liver diseases includes radical species, including reactive oxygen species (ROS), known as by-products of oxygen metabolism, and reactive nitrogen species (RNS) derived from nitric oxide and superoxide (Li et al., 2015; Ramos-Tovar and Muriel, 2020). In normal physiological conditions, ROS are involved in destroying microorganisms and several signal transduction pathways, including gene expression (Li et al., 2015). However, high ROS/RNS levels can damage various biological molecules such as DNA, proteins, and lipids, resulting in disease (Li et al., 2015). Oxidative stress is an imbalance between ROS generation and elimination (Li et al., 2015; Sharifi-Rad et al., 2020), resulting in several disorders, including cancer, diabetes, and liver pathologies (Ramos-Tovar and Muriel, 2020). Antioxidants are molecules from endogenous or exogenous sources used to prevent or reduce the extent of ROS damage (Ramos-Tovar and Muriel, 2020). Medicinal plants are a significant source of natural antioxidants that have been reported to have beneficial effects on many ailments, including liver diseases (Agbor et al., 2022; Li et al., 2015; Ramos-Tovar and Muriel, 2020). Therefore, studying plants or herbs susceptible to protecting the liver or other organs may help to treat liver ailments. Among medicinal plants with hepatoprotective effects are *Balanites aegyptiaca* (L.) Delile. (Zygophyllaceae), *Calotropis procera* (Aiton) Dryand. (Apocynaceae), *Cochlospermum tinctorium* Perrier ex. A.Rich. (Bixaceae), and *Acanthospermum hispidum* DC. (Asteraceae) (Figure 1). In Burkina Faso traditional medicine, these plants are used as depurative, diuretic, and to treat liver disorders (Nacoulma Ouedraogo, 1996).

Commonly named "Desert date," *B. aegyptiaca* is a spiny shrub or tree belonging to the family of Zygophyllaceae (Chothani and Vaghasiya, 2011). Native to Africa, *B. aegyptiaca* is also found in parts of the Middle East, Western, and South Asia (Murthy et al., 2021). In African and Indian traditional medicine, different parts of this plant are used to treat several ailments, including jaundice, chronic hepatitis, malaria, edema, hemorrhoids, and helminthiasis (Murthy et al., 2021; Traore et al., 2018). *B. aegyptiaca* contains various phytochemicals: saponins, polyphenols, coumarins, and alkaloids (Murthy et al., 2021). Several published papers have reported some of its biological activities, including antioxidant, antimicrobial, hepatoprotective, and anticancer (Traore et al., 2020; Murthy et al., 2021).

C. procera is a shrub that belongs to the family of Apocynaceae. The plant is widespread worldwide and grows in various environments, including warm climates, landfills, roadside ditches, and wastelands (Dogara, 2023). Known as Sodom's apple, *C. procera* is widely used in folk medicines to treat many diseases like rheumatism, asthma, fever, eczema, diabetes, and malaria (Wadhwani et al., 2021; Dogara, 2023). The pharmacological studies have demonstrated its several biological properties, including antioxidant, anticancer, antibacterial, antiulcer, anti-inflammatory, antidiabetic, and hepatoprotective (Al-Rowaily et al., 2020; Wadhwani et al., 2021; Dogara, 2023). Numerous phytochemicals, such as terpenoids, flavonoids, tannins, steroids, alkaloids, and saponins, have been found in different plant parts (Al-Rowaily et al., 2020).

In several parts of African countries, *C. tinctorium* is widely used in traditional medicine. Roots, rhizomes, leaves, and flowers are used to cure many ailments, such as convulsion, inflammation, diabetes, ulcers, and liver diseases (Ahmad et al., 2021). *C. tinctorium* contains alkaloids, flavonoids, tannins, glycosides, lignans, peptides, and proteins. The demonstrated pharmacological activities of this sub-shrub include antioxidant, antihelminthic, antiplasmodial, antimicrobial, anti-inflammatory, hepatoprotective, and anticonvulsant (Ahmad et al., 2021). Native to Northern South America, *A. hispidum* is present in other parts of the world, such as Africa, India, Hawaii, and Brazil (Dos Santos et al., 2022; Patel et al., 2022). Leaves, roots, and the whole plant are used to alleviate various diseases, including bronchitis, arthritis, jaundice, malaria, and liver diseases (Patel et al., 2022). The biological activities of *A. hispidum* have been well described; among these are antimicrobial, antioxidant, anticholinesterase, and hepatoprotective (Dos Santos et al., 2022). Various phytochemicals have been detected in *A. hispidum*: terpenes, steroids, tannins, and flavonoids (Dos Santos et al., 2022).

Based on the scientific data and the traditional uses of these four plants, mostly against liver diseases, it was hypothesized that a recipe with a combination of these plants would be more powerful at protecting the liver than each plant. Therefore, the present report aimed to assess the antioxidant and antiproliferative potentials and determine the polyphenol contents of a recipe made of *B. aegyptiaca*, *C. procera*, *C. tinctorium*, and *A. hispidum*. To our knowledge, this is the first report investigating the biological activities of Burkina Faso's plant mixture composed of these four plants. The results could serve to develop a potent plant formulation against liver diseases

MATERIALS AND METHODS

Solvents, reagents and equipment

Methanol and ethanol were from Carlo Erba (France). Dimethyl

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1. *Balanites aegyptiaca*

2. *Calotropis procera*

3. *Acanthospermum hispidum*

4. *Cochlospermum tinctorium*

Figure 1. Representative pictures of the four plants used in the mixture.

sulfoxide (DMSO), Folin and Ciocalteu Reagent (FCR), sodium carbonate ascorbic acid, gallic acid, quercetin, trolox, 2-aminoethyl diphenylborinate, aluminium trichloride ($AlCl_3$), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical were supplied by Sigma-Aldrich (USA). Dulbecco's Modified Eagle's Medium (DMEM, 10-013-CV) and trypsin EDTA 1X, and 0.05% (25-051-CI) were purchased at Corning GmbH (Germany). All other chemicals and reagents were either from analytical or technical grade.

Sterile 96-well microplates treated for cell culture were obtained from Falcon, 353072, USA, and the ATPlite luminescent assay system (6016947) was from PerkinElmer (France). Thin-layer chromatographic (TLC) plates from Merck (Germany) were used to assess the phytochemical composition of the recipe. Three spectrophotometers were used to measure absorbances: Agilent 8453 UV-visible (USA), Model 680 microplate reader (BIO-RAD, Belgium), and a Fluoroskan FL (ThermoFisher Scientific, France).

Plant: Collection and extraction

The whole plant of *A. hispidum* was collected at Dedougou (N0441167, W1364395), a city in western Burkina Faso. The stem barks of *C. procera* were harvested at Gampela (N1225282, W1280060), a peri-urban area around Ouagadougou. Root barks of *B. aegyptiaca* were from Boromo (N0510253, W1268027), a city of the Boucle du Mouhoun region located between Ouagadougou and Bobo-Dioulasso, the two major cities of Burkina Faso. Root barks of *C. tinctorium* were obtained at Farako-ba (N0354870, W1227579), a peri-urban area of Bobo-Dioulasso. Plant names were confirmed using the website http://www.worldfloraonline.org. A botanist (Dr. Compaore Souleymane) authenticated the collected parts by comparing them to specimen vouchers deposited at the herbariums of the University Joseph Ki-Zerbo (N°6916 for *B. aegyptiaca*; N°6980 for *C. tinctorium*; N°16875 for *A. hispidum*) and National Center for Scientific and Technological Research (CNRST) (N°HB 8716 for *C. procera*).

The plant parts were shade-dried for at least two weeks. After that, they were powdered using a grinder. To prepare the medicinal recipe, 100 g of each powdered plant part was placed in a container, and 1 L of 96% ethanol was added. The mixture was left to macerate under mechanical agitation for 24 h at room temperature. Then, it was filtrated, concentrated, and lyophilized (BIOBASE, China). The extraction's yield was determined by applying the following equation:

$$Yield\ (\%) = \frac{\text{Weight of the lyophilized extract}}{\text{Weight of the initial dried sample}} \times 100$$

Phytochemical analysis

Qualitative and quantitative methods were used to assess the phytochemical composition of the recipe. A modified methodology

was used to perform the qualitative phytochemical content of the recipe (Ciulei, 1982). Tannins, flavonoids, alkaloids, saponins, sterols, triterpenes, anthocyanins, coumarins, and reducing sugars were researched in the recipe. Thin-layer chromatography was used to confirm the presence of the phytoconstituents using specific revelators.

The quantitative method consists of estimating the total phenolic and flavonoid contents in the recipe. The total phenolic content (TPC) was estimated according to the method described by Singleton et al. (1999) with some modifications. In brief, 0.25 ml of a 10 mg/ml ethanolic extract was mixed with 0.25 ml of 2 N Folin and Ciocalteu's phenol reagent. After 3 to 10 min at room temperature, 0.75 ml of 20% sodium carbonate was added. The mixture was allowed to stand for 40 min; after that, absorbance was recorded at 760 nm (Agilent 8453 UV-visible spectrophotometer). In the control tube, the recipe was replaced with distilled water. TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract (mg GAE/g).

The total flavonoid content (TFC) was determined using a modified method described by Abdel-Hameed (2009). To 0.6 ml of a 1 mg/ml ethanolic extract, 0.6 ml of 2% aluminium trichloride was added. The mixture was well homogenized and incubated at room temperature for 40 min. Then, absorbance was read at 415 nm using the Agilent 8453 UV-visible spectrophotometer. The blank was performed with 0.6 ml of $AlCl_3$ and 0.6 ml of methanol. Quercetin served as standard, and TFC was expressed as milligrams of quercetin equivalent (QE) per g of dry extract (mg QE/g).

Evaluation of antioxidant potential

The antioxidant potential of the recipe made of the four plants was assessed using four methods: radical scavenging (DPPH and ABTS assays), ferric-reducing antioxidant power (FRAP), and lipid peroxidation (LPO).

DPPH radical scavenging effect

The radical scavenging activity of the recipe was assayed on DPPH radicals using the method of Kim et al. (2007) with some modifications. 20 µL of the recipe and Trolox at different concentrations were mixed with 200 µL of DPPH solution at 0.04 mg/ml in a 96-well plate. The plates were left to incubate in the dark at room temperature for 30 min. The absorbance was read at 490 nm using the BIO-RAD spectrophotometer. In the control wells, the sample was replaced by methanol.

The DPPH radical scavenging effect was calculated from the following equation:

$$DPPH\ \text{scavenging activity}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

ABTS radical scavenging activity

A modified method from Re et al. (1999) was used to determine the recipe's and Trolox's scavenging effect on ABTS radicals. The ABTS radicals were produced by reacting the ABTS (7 mM) salt with potassium persulfate (2.45 mM). The mixture was allowed to incubate in the dark for 12 to 16 h at room temperature. For the experiment, 20 µL of different concentrations of the recipe and Trolox, prepared from a 1 mg/ml stock solution, were added to 200 µl of ABTS solution in a 96-well microplate. The control consists of 20 µl of ethanol and 200 µl of ABTS solution. Then, the plates were incubated in the dark at room temperature for 30 min. The absorbance was measured at 415 nm (BIO-RAD spectrophotometer). The ABTS scavenging activity was obtained using the following equation:

$$ABTS\ scavenging\ activity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Ferric-reducing antioxidant power (FRAP) assay

The reducing power of the recipe was evaluated according to the method of Hinneburg et al. (2006). Briefly, 0.5 ml of a 1 mg/ml recipe was mixed with 1.25 ml phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min, then mixed with 1.25 ml of 10% trichloroacetic acid and centrifuged for 10 min at 3000 rpm. In a glass tube, 0.625 ml of the supernatant, 0.625 ml of distilled water, and 0.125 ml of 1% ferric chloride were mixed. Afterward, the absorbance was taken at 700 nm. Ascorbic acid was used as the standard, and the reducing power was expressed as mol ascorbic acid equivalent (AAE) per g of recipe (mol AAE/g).

Lipid peroxidase inhibition (LPO)

The capability of the recipe to inhibit lipid peroxidation was performed using a rat liver homogenate according to a previous method (Sombie et al., 2011). The Trolox was used as a control. The reaction mixture comprised 72 µl of the recipe (1 mg/ml) and 360 µl of liver homogenate. Then, 18 µl of 0.5 mM ferrous chloride and 18 µl of 0.5 mM hydrogen peroxide were added. The mixture was incubated at 37°C for 1 h, followed by the addition of 360 µl of 15% trichloroacetic acid and 360 µl of 0.67% thiobarbituric acid. After 15 min in a boiling water bath, the absorbance was measured at 550 nm using the BIO-RAD spectrophotometer. The following formula determined the percentage of the inhibition (I%) of lipid peroxidation:

$$I\ (\%) = \left[1 - \left(\frac{A1 - A2}{Ac}\right)\right] \times 100$$

where Ac represents the absorbance of the control (liver homogenate and reagents without sample), A1 is the absorbance of the sample, and A2 is the absorbance without the liver homogenate.

Evaluation of the antiproliferative activity

The antiproliferative effect of the recipe was assessed using the chemoresistant human hepatocarcinoma cell line Hep3B. Hep3B cells (HB-8064TM, ATCC, USA) were cultured in a DMEM medium containing 10% fetal bovine serum, non-essential amino acids, and sodium pyruvate. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. When reaching 70 to 90% confluence, the Hep3B cells were washed with phosphate-buffered saline (PBS 1X), trypsinized, and subcultured. Hep3B cells were seeded at 5,000 cells per well in a 96-well plate 24 h before adding the recipe. The recipe was tested at 0.1, 1, 2.5, 5, 10, 25, and 50 µg/ml concentrations containing 0.1% of DMSO as the vehicle. The antiproliferative activity was measured 48 h after using an ATP-based luminescence assay (ATPliteTMkit, Perkin-Elmer, France) according to the method of Carraz et al. (2015). The results were compared with controls at 0.1% DMSO. Experiments were performed in triplicate.

Hep3B cells morphological analysis

Hep3B cells were seeded into six-well culture plates (Falcon, USA)

Table 1. Qualitative and polyphenol contents of the recipe.

Phytochemical tests	Recipe
Extraction yield (%)	7.56
Qualitative analysis	
Tannins	+
Alkaloids	-
Saponins	+
Anthocyanins	-
Flavonoids	+
Sterols	+
Triterpenes	+
Coumarins	+
Cardenolides	-
Reducing sugars	+
Quantitative analysis	
TPC (mg of GAE/g of extract)	208.81 ± 1.13
TFC (mg of QE/g of extract)	10.74 ± 1.44

Signs (+) and (-) indicate the presence or absence of a phytochemical. TPC: Total phenolic content; TFC: total flavonoid content.

at a density of 5 × 104 cells. After 24 h, cells were treated with fresh medium containing either the IC_{50} value for the recipe (1.1 µg/ml) or approximately half or twice the IC_{50} (0.5 or 2 µg/mL, respectively). The untreated cells (Control) received an equal medium volume with 0.1% DMSO. The cells (treated and untreated) were examined at 24 and 48 h. Cell images were obtained using an inverted phase contrast microscope (Zeiss, USA) at 400 × magnification.

Data presentation and statistical analysis

Using DPPH and ABTS radicals, the radical scavenging effect was expressed as IC_{50} values, representing the inhibitory concentration that scavenges 50% of radicals. Similarly, the ability of the recipe to inhibit the proliferative activity of the recipe was expressed as an IC_{50} value. IC_{50} values were estimated using GraphPad Prism (version 8.0). The experiments were repeated thrice, and the data were presented as means ± standard deviation (SD). An unpaired t-test was used to compare the recipe to the standard. Statistical differences were considered significant when p-value < 0.05.

The Pearson coefficient (r) (Ratner, 2009) was used to evaluate the relationships between the *in vitro* activities and the polyphenol content. The coefficients were obtained using GraphPad Prism 8.

RESULTS

Qualitative and quantitative phytochemical analysis

The results of the extraction yield, qualitative, and quantitative phytochemical analysis of the recipe are shown in Table 1. Using the standards protocols and the TLC analysis, the following phytochemicals are revealed in the recipe: tannins, saponins, sterols, triterpenes, flavonoids, coumarins, and reducing sugars. However, alkaloids, anthocyanins, and cardenolides were absent. Moreover, the quantitative estimation of polyphenols indicates that the recipe is rich in these compounds, although phenolics seem more abundant than flavonoids.

Antioxidant and antiproliferative effects of the recipe

Table 2 shows the results of the effect of the recipe on the different antioxidant models. The antioxidant activity of the recipe was measured through IC_{50} values, FRAP values (in mol AAE/g), and the percentage of inhibition for the lipid peroxidation assay. Considering the radical scavenging assays, the recipe was more potent on ABTS than DPPH radicals. However, the radical scavenging ability was lesser than Trolox. The FRAP assay indicates that the recipe has a high-reducing power. In the lipid peroxidation assay, the recipe significantly inhibited the malondialdehyde levels compared to the Trolox.

The ATPlite assay was used to measure the antiproliferative effect of the recipe. The recipe significantly inhibits the proliferation of Hep3B cells compared to the control cells 48 h after incubation. The IC_{50} value was 1.1 ± 0.75 µg/ml. To confirm the ATPlite data, the Hep3B cells were treated for 48 h with the recipe at 0.5, 1.1 (IC_{50} value), and 2 µg/ml. Figure 2A and B shows the images of untreated and treated Hep3B cells at 24 and 48 h, respectively. Compared to the untreated cells, it can be noticed that the recipe provokes significant changes in the Hep3B cells in a dose-dependent manner.

Table 2. Effect of the recipe on the different antioxidant models.

Sample	IC_{50} (µg/mL)		FRAP values	Lipid peroxidation (%)
	DPPH	ABTS		
Trolox	0.006 ± 0.0001	0.003 ± 0.0002	-	48.11 ± 3.88
Recipe	328.31 ± 0.08****	11.61 ± 0.0006****	133.06 ± 2.09	56.39 ± 2.55*

Values are mean ± standard deviation of three measurements. ****p < 0.0001, *p < 0.05 vs. Trolox.

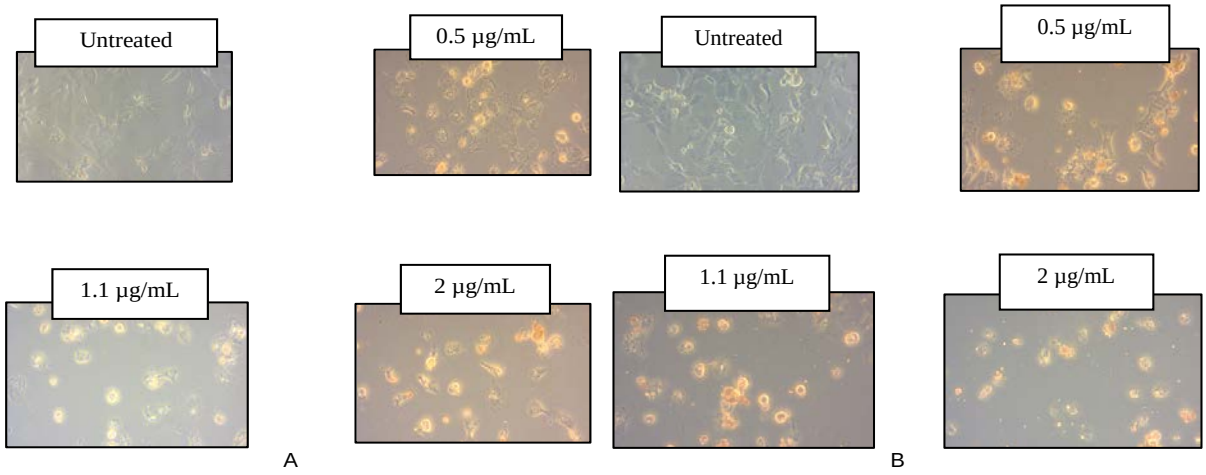

Figure 2. Hep3B cell images (A) 24 h and (B) 48 h after treatment or not with the recipe. The treated cells received the recipe at 0.5, 1.1 (IC_{50}), and 2 µg/ml.

Table 3. Correlation matrix between polyphenol contents (TFC and TPC) and the antioxidant and antiproliferative effects.

Test	FRAP	DPPH	ABTS	LPO	TFC	TPC	ATPlite
FRAP	1						
DPPH	0.738	1					
ABTS	-0.997	-0.791	1				
LPO	0.875	0.972	-0.912	1			
TFC	0.441	-0.281	-0.365	-0.049	1		
TPC	-0.796	-0.178	0.743	-0.403	-0.894	1	
ATPlite	-0.636	0.052	0.570	-0.183	-0.973	0.973	1

The effect was noticeable 24 h after treatment, and 48 h after, there were more dead cells. The results confirm the cytotoxicity potency of the recipe. Almost 90 to 95% of the cells were destroyed at 1.1 and 2 µg/ml concentrations 48 h after treatment. The remaining live Hep3B cells at 0.5 µg/ml concentration were almost altered 72 h after treatment (data not shown).

Correlations between the polyphenols contents and antioxidant and antiproliferative activities of the recipe

The results showed a weak to moderate correlation between the different tests and total flavonoid content (Table 3). The correlation was negatively high between TPC and FRAP assay; however, the recipe presents a high positive correlation between the TPC and their potential to scavenge ABTS radicals (r = 0.743) and inhibit the proliferation of Hep3B cells (r = 0.973).

DISCUSSION

The liver plays a key role in the body; therefore, studies aiming to protect it from hepatotoxins are beneficial. *B. aegyptiaca*, *C. procera*, *C. tinctorium*, and *A. hispidum* are four plants used in traditional medicine against

various ailments, including hepatic diseases. The present study examined the qualitative phytochemical and polyphenol content, the antioxidant and antiproliferative effects of a recipe made of four medicinal plants, including *B. aegyptiaca*, *C. procera*, *C. tinctorium*, and *A. hispidum*. By using maceration, the extraction yield was 7.56%. This extraction method was preferred based on the unknown nature, labile or thermolabile, of the compounds in the mixture. Furthermore, this method has been used to extract various phytoconstituents, including phenolics (Hidayat and Wulandari, 2021). The qualitative phytochemical analysis of the recipe showed the presence of various secondary metabolites such as tannins, saponins, flavonoids, sterols, triterpenes, and coumarins. Alkaloids, anthocyanins, and cardenolides were absent. The phytochemical investigation indicated approximately a similar composition of phytochemicals in each plant. Indeed, it was reported that various saponins and steroids were isolated from the root extracts of *B. aegyptiaca*; moreover, tannins, triterpenes, flavonoids, coumarins, and steroids were present in different parts of the plants: *C. procera*, *C. tinctorium*, and *A. hispidum* (Ahmad et al., 2021; Al-Rowaily et al., 2020; Wadhwani et al., 2021; Dofuor et al., 2022; Dos Santos et al., 2022; Murthy et al., 2021). Alkaloids were detected in the aqueous methanol extract of the root bark of *C. tinctorium* (Ahmad et al., 2021) but not in the present study, suggesting that other compounds in the plant's mixture may be present but at lower levels, so undetectable with the current phytochemical analysis. The quantitative content analysis indicated that the plant's mixture was rich in polyphenol compounds, confirming that the mixture with the four plants did not affect the polyphenol contents. Indeed, each plant contains variable quantities of polyphenol compounds (Ahmad et al., 2021; Dogara, 2023; Murthy et al., 2021; N'do et al., 2018).

Numerous compounds, including phenolic acids, flavonoids, tannins, saponins, steroids, and coumarins, have displayed antioxidant activity (El-Lateef et al., 2023; Murthy et al., 2021). It is well-reported that using two or more antioxidant test methods provides more comprehensible details on a sample's antioxidant potential than a single one (Genskowsky et al., 2016). In the current study, four antioxidant methods, including radical scavenging, lipid peroxidation, and ferric-reducing assays, were used to assess the antioxidant potential of the plant's mixture. The mixture displayed a poor radical scavenging effect compared to the trolox. A more potent effect on the $ABTS^{\bullet+}$ radicals than DPPH ones has been observed. Interestingly, compared to trolox, the plant's mixture was more active on malondialdehyde (MDA), indicating an interesting antioxidant capacity for lipid peroxidation.

Similarly, the plant's mixture was potent in reducing ferric iron to ferrous iron, showing that the ethanolic extract possesses high concentrations of reducer compounds. Previous reports on the four plants composing the mixture have demonstrated the antioxidant potential of each plant (Dogara, 2023; Murthy et al., 2021; N'do et al., 2018; Ndouyang et al., 2018). However, this effect may be lesser or higher than an individual plant's antioxidant effect. For instance, the ethanolic effect of *A. hispidum* was more potent on the DPPH, ABTS, and FRAP assays (N'do et al., 2018) than the plant's mixture. The differences between individual plants and the mixture may be related to their phytochemical nature and contents. In fact, herbal mixtures contain various compounds with synergistic, additive, and antagonistic activity (Caesar and Cech, 2019). Therefore, these results suggest that some compounds may act as inhibitors of other compounds, leading to poor bioactivity. Various phytochemicals, including polyphenols, coumarins, and saponins, may explain the antioxidant potential of the mixture. The correlation study indicates that total flavonoids were not correlated with any antioxidant assay, suggesting that other phytochemicals may account for the observed antioxidant activity. The correlation between the ABTS test and total phenolic was highly positive, suggesting that the phenolics are presumably responsible for the ABTS radical scavenging effect. However, phenolics were negatively correlated with the FRAP assay. This result suggests that the high ferric iron-reducing power may be due to other compounds, such as saponins, coumarins, and steroids, that can act as antioxidants (El-Lateef et al., 2023; Murthy et al., 2021). Since free radicals are implicated in liver disorders, the results of the present study show that the mixture can, to some extent, protect the liver by inhibiting ROS production. Therefore, further studies regarding inhibiting intracellular ROS on various liver cell lines will be helpful.

The plant's mixture was evaluated on the human hepatocarcinoma Hep3B cells to determine its antiproliferative effect. Results showed a significant and dose-dependent inhibition of the proliferation of this cell line. The microscopic images of Hep3B cells treated at the IC_{50} value and 2 µg/ml concentrations confirmed the high cytotoxicity potential of the recipe. Based on the US National Cancer Institute (NCI), the mixture may be seen as a potent cytotoxic product since the IC_{50} value was below 30 µg/ml (Alonso-Castro et al., 2011). The anticancer or antiproliferative effects of *B. aegyptiaca*, *C. procera*, *C. tinctorium*, and *A. hispidum* have already been reported (Ahmad et al., 2021; Hassan et al., 2016; Bou Malhab et al., 2023; Ibrahim et al., 2022; Mathur et al., 2009; Rajendran and Deepa, 2007). Consequently, the mixture of these four plants did not affect their antiproliferative activity. To our knowledge, no anticancer or antiproliferative effect of each plant on the Hep3B cells has been reported. At least 40% of anticancer drugs discovered from 1940 to 2014 were derived from natural products (Choudhari et al., 2019). According to these authors, various phytochemicals, including phenolics, flavonoids, triterpenes, saponins, and steroids, are involved in the anticancer effects of medicinal plants (Choudhari et al., 2019; Ibrahim et al., 2022). A high

positive correlation (r = 0.973) was obtained between the ATPlite assay and total phenolic content, suggesting that these compounds account for the antiproliferative potential of the mixture. The anticancer mechanisms of phytochemicals are various and involve (i) free radicals scavenging, (ii) suppression of survival and proliferation of cancerous cells, and (iii) inhibition of multiple molecular targets and signal transduction pathways involved in tumorigenesis (Choudhari et al., 2019; Ranjan et al., 2019). Free radicals are involved in developing several diseases, including cancer. Results of the present study suggest that the mixture may be considered as a chemoprotective product. Many preclinical reports have been dedicated to the chemopreventive effect of phytochemical compounds. Phytochemicals such as resveratrol and curcumin have been investigated for their potential as chemoprotective agents (Boretti, 2022; Xiao et al., 2018). However, most studies were preclinical since clinical trials gave negative or null results (Boretti, 2022). Findings of the present study pave the way for future inquiries to increase our understanding of this plant mixture's chemoprotective mechanism of action. Further detailed investigations will lay the foundations for a robust clinical trial with the plant's mixture.

Conclusion

There is strong evidence that synergistic combinations of two or more compounds or herbal extracts may improve efficacy. These studies have gathered the attention of various scientific research teams, including ours. In this paper, we report for the first time, to the best of our knowledge, the antioxidant and antiproliferative properties of a recipe composed of *B. aegyptiaca*, *C. procera*, *C. tinctorium*, and *A. hispidum* medicinal plants used in traditional medicine to treat liver disorders. The plant's recipe contains various phytochemicals, including polyphenols, steroids, saponins, and triterpenes. The mixture developed potent *in vitro* antioxidant and antiproliferative activities. These significant effects may be due to the total phenolic content. Findings of the this study demonstrate that the mixture of four plants with hepatoprotective potential can help to protect the liver and inhibit the proliferation of the human hepatocarcinoma cell line Hep3B. Thus, these results encourage us to continue investigating this plant recipe combination's antioxidant and antiproliferative mechanisms of action.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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