



(RESEARCH ARTICLE)



Phytochemical analysis, antioxidant and anti-inflammatory activities of *Chassalia kolly* leaves extract, a plant used in Benin to treat skin illness

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GSC Biological and Pharmaceutical Sciences, 2021, 15(03), 063–072

Publication history: Received on 28 April 2021; revised on 30 May 2021; accepted on 04 June 2021

Article DOI: <https://doi.org/10.30574/gscbps.2021.15.3.0148>

Abstract

In the last decades, it has been intensively studied natural alternatives to synthetic products. *Chassalia kolly* is one of the medicinal plants used traditionally for treatment of skin infections. This study aimed at identifying the diversity secondary metabolites, to investigate the antioxidant and anti-inflammatory activities of ethanolic extract of *Chassalia kolly* leaves. Total phenols were determined by Folin Ciocalteu method. The aluminum trichloride method has been used to quantify total flavonoids, while the determination of condensed tannins was carried out by the hydrochloric vanillin method. The antioxidant capacity was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-Azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) and phosphomolybdenum methods. *In vitro* anti-inflammatory activity of ethanolic extract of *Chassalia kolly* leaves was evaluated by method membrane stabilization. In this results, *Chassalia kolly* leaves contain flavonoid, mucilages, anthocyanins, sterols and terpenes, saponosides, alkaloids and reducing compounds. The contents of total phenols, total flavonoids and total tannins are respectively (20.41±2.11) µgGAE/mgDM; (30.29±2.18) µgQE/mgDM and (1.211±0.122) µgCE/mgDM. The results obtained during this work allowed us to assert that all extracts of the studied plant have very good antioxidant properties (IC₅₀=0.05µg/µL). The ethanolic extract of the leaves of *Chassalia kolly* showed an anti-inflammatory activity more interesting than aspirin which is the reference compound used in this study.

Keywords: Skin; *Chassalia kolly*; secondary metabolites; antioxidant; anti-inflammatory

1. Introduction

Infectious diseases are one of the major causes of death in tropical countries. For a long time, chemotherapy based on the use of antibiotics remained the fastest and most effective way to treat infections. Unfortunately, the upsurge of microbial resistance to antibiotics and the spread rate of resistant bacterial species became major public health concern. In recent years, research has turned to therapeutic alternatives to prevent the upsurge from bacterial resistance to existing molecules. African flora in general and Benin in particular, abounds in an important reserve of medicinal plants. Thus, medicinal plants occupy an important place in the African pharmacopoeia. Even nowadays, medicinal plants have been used for treatments of various diseases since antiquity and still play an important role to cover the basic health needs in the developing countries [1]. Skin is larger than any other organ in humans. Like other organs, various bacterial, viral, and inflammatory diseases, as well as cancer, affect the skin. Skin diseases like acne, atopic dermatitis, and

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psoriasis often reduce the quality of life seriously [2]. Skin diseases are often not considered in priority areas of health systems planning, due in part to the infrequent mortality, and also to the lack of awareness of the burden of skin diseases in public health terms [3]. Skin diseases are abundant and diverse, accounting for about one third of medical consultations of Africa [4]. Skin diseases affect 21% to 87 % of children in African developing countries and constitute up to a third of outpatient visits to Pediatricians and Dermatologists [5], [6]. Despite their common occurrence, skin diseases receive less attention as compared with diseases such as malaria, pneumonia and HIV/AIDS, which cause significant mortality [7], [8]. However, the spectrum of the skin diseases varies from region to region due to several factors such as genetical, socioeconomical and environmental field [8]. Most of the people in rural and urban areas of the developing world depended on the medicinal plants for the treatment of infectious diseases, in particular the infections of skin. Therefore, effective treatment of skin disorders is important. *Chassalia kolly* the family Rubiaceae, is a soft-stemmed shrub native to West Africa [9]. *Chassalia* species have been prescribed for wound dressing, inflammation and liver disease, as an insect repellent and skin diseases in traditional medicine in Africa [10]. The studies on biological activities of medicinal plants and search for source of new therapeutic drugs, we now report on insecticidal, antimicrobial, phyto- and cytotoxicity of *Chassalia kolly*, whole plant extract [11]. The aim of this study is to promote *Chassalia kolly*, a plant used in Benin to treat skin infections by identifying secondary metabolites and then evaluating antioxidant and anti-inflammatory activities.

2. Material and methods

2.1. Materials

2.1.1. Plant material

Chassalia kolly leaves used in this study were collected from Abomey-Calavi in Benin.

2.1.2. Chemicals

Methanol, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, gallic acid, aluminum chloride, potassium acetate, sodium acetate, ascorbic acid, aspirin, catechin, trolox, ammonium molybdate, ascorbic acid, hydrochloric acid, sulfuric acid and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) were purchased from Sigma-Aldrich. All reagents and chemicals were analytical grade.

2.2. Methods

2.2.1. *Chassalia kolly* leaves pretreatment

After harvesting, the samples were dried at laboratory temperature until their plant mass stabilized and then reduced to powder.

2.2.2. Plant extracts

The extraction was made with ethanol and hydroethanolic under ultrasounds. Briefly, 10 g of powdered biomass were mixed with 100 mL solvent and sonicated for two hours at 50°C with Bandelin (Sonorex Digitech device). Further, all the extracts were filtered through Whatman No.1 filter paper and concentrated under vacuum (Buchi R215, heating bath B-491, rotation 280 rpm, vacuum controller V-850 of 290 mbar) at (50±1) °C. The residues were dried to constant weights and stored in the darkness at 4°C to avoid the degradations until use [12], [13], [14].

2.2.3. Preliminary phytochemical screening

Secondary metabolites were carried out by coloration and precipitation reactions specific to each family of metabolites [15-17].

Table 1 Methods for the identification of secondary metabolites of *Chassalia kolly* leaves

Secondary metabolites	Chemical test
Alkaloids	Mayer's test and Drangendorffs test
Anthocyanins	test with hydrochloric acid and ammonia
Antraquinones	Borntranger's test
Coumarins	365 nm , fluorescence test
Flavonoids	Shibita's reaction test
Tannins	stiasny test, ferric chloride and sodium acetate test
Saponin	Frothing test
Leuco anthocyanins	Bate-Smith and metcalf
Mucilage	flaky test
Cyanogenic derivatives	picric acid test
Reducing compound	Fehling's test
Sterols and terpenes	Liebermann-Burchard's test)

2.2.4. Determination of polyphenolic compounds

Total phenol content

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method. This method consisted of using a mixture of phosphotungstic and phosphomolybdic acids, which were reduced during the oxidation of phenols into a mixture of tungsten blue oxide and molybdenum. Finally, the absorbance was measured at 760 nm using a spectrophotometer (with Infinite 200 PRO-Tecan microplate) and the total phenol content are expressed in micrograms of gallic acid equivalence per milligram of dry matter ($\mu\text{gGAE}/\text{mgDM}$) [12], [18], [19].

Total flavonoids content

The method of aluminum trichloride (AlCl_3) was used to quantify the total flavonoids. This technique was based on the formation of the aluminum complex flavonoids. The absorbance was read at 415 nm using a spectrophotometer (Infinite 200 PRO-Tecan microplate) and the Total flavonoid content are expressed in micrograms quercetin equivalence per milligram of dry matter ($\mu\text{gQE}/\text{mgDM}$) [20].

Condensed tannin content

Vanillin and hydrochloric acid method was used to determine the total condensed tannins content. The absorbance were measured at 500 nm using the spectrophotometer (Infinite 200 PRO-Tecan microplate) and the tannin content was expressed in micrograms catechin equivalence per milligram dry matter ($\mu\text{gEC}/\text{mgDM}$) [21].

2.2.5. Antioxidant activity

The antioxidant activity of the ethanolic extract of *Chassalia kolly* leaves evaluated by ABTS, DPPH and ammonium molybdate tests.

2.2.6. DPPH free radical scavenging assay

100 μL of ethanolic extract of *Chassalia kolly* leaves were added to the five wells of the first two 96-well microplate lines. Then a gradual dilution of a ratio of two was carried out starting with the second line with methanol until the last line (8th line) where 100 μL of dilution was discarded at each well. In the first three wells of each row, 100 μL of the methanolic solution of DPPH (0.1mg/mL) were added and 100 μL of methanol in the other, two wells of each row to prepare the negative control. The positive control is prepared in parallel by mixing 100 μL of methanol with 100 μL of the DPPH solution. After incubation in the dark room the temperature, the absorbance is measured after 15 minutes for one hour at 517 nm using the spectrophotometer (microplaque Infinite 200 PRO-Tecan)[12],[19],[22].

$$P(\%) = \frac{[A_p - (A_s - A_n)]}{A_p} \times 100$$

A_p : Positive control absorbance; A_n : Negative control absorbance; A_s : sample absorbance

2.2.7. Phosphomolybdenum assay

100 μ L of ethanolic extract of *Chassalia kolly* at different concentrations were added to 1000 μ L of a reagent composed of sulfuric acid (0.6M), sodium phosphate (28mM) and ammonium molybdate (4mM). The tube was incubated at 95°C for 90 minutes and after cooling, the absorbance was measured at 695nm. The control consists of 100 μ L of dissolution solvent mixed with 1000 μ L of the reagent mentioned above. Samples and controls are incubated under the same conditions and then the absorbance is measured using a spectrophotometer (Infinite 200 PRO-Tecan). The results obtained are expressed in micrograms ascorbic acid equivalent per milligram of dry matter of the extract (μ gAAE/mgDM) [23].

2.2.8. ABTS free radical scavenging assay

The potential of the extracts to reduce the ABTS⁺ radical was evaluated in using the method described by Miller et al [23]. The principle is based on the ability of an antioxidant to stabilize the blue-green cationic radical ABTS⁺ by transforming it into colorless ABTS[•] by trapping a proton. The cationic radical ABTS⁺ was obtained from 10 ml of ABTS (2mM) and 100 μ L of potassium persulfate (70 mM). The mixture was stored in the darkness for 6 hours before performing the test. 100 μ L of extract was added to 100 μ L of ABTS⁺ and the absorbance was measured at 734 nm after 15 min for 1 hour. The blank was prepared by mixing 100 μ L of extract with 100 μ L of ethanol with a control or 100 μ L of methanol was mixed with 100 μ L of the ABTS⁺ radical. The potential of the extracts to reduce the ABTS⁺ radical was expressed in microgram equivalence of Trolox per milligram of dry matter (μ gETx/mgDM) from the calibration line of the Trolox [12],[19], [22].

2.2.9. Anti-inflammation activity

Anti-inflammatory activity was assessed by determining the stabilizing potential of the membrane of red blood cells. For this, 100 μ L of fresh human blood were mixed with 900 μ L of sodium chloride solution (0.9%) and then centrifuged at 8000 rev/min for 10min. Then 300 μ L of supernatant was added to 300 μ L of extract and stirred for 30 min. For the negative control, 300 μ L of sodium chloride solution was mixed with 300 μ L of supernatant. Aspirin was used as a reference compound by mixing 300 μ L of aspirin (8 μ g/ μ L) and 300 μ L of methanol. The samples were incubated at 56 °C for 30 min, centrifuged at 2500 rpm for 5min and the absorbance of the supernatant was measured at 560 nm. The experiment was carried out in triplicating. The percentage of stabilization of the red blood cell membrane was calculated [24], [25] [26].

$$PP = \frac{100 - (A_e - A_b)}{A_{cm}} \times 100$$

A_e : Sample absorbance; A_b : Absorbance of white; A_{cm} : Absorbance of negative control

3. Results and discussion

3.1. Secondary metabolites in the *Chassalia kolly* leaves

The secondary metabolites identified in the leaves of *Chassalia kolly* are listed in Table 2. The results of the preliminary phytochemical screening revealed the presence of glycosides, alkaloids, flavonoids, anthocynes, reducing compound, mucilages and saponosids in the *Chassalia kolly* leaves. In the *Chassalia kolly* leaves collected from the Ibadan (Nigeria) glycosides, alkaloids and flavonoids have been identified [27]. The variation of secondary metabolites observed at the level of our samples compared to previous work could be related to the harvest period, the nature of the soil or climatic factors [28], [29]. The use of *Chassalia kolly* in traditional medicine to treat skin infections could be explained in particular by the presence of mucilages, saponosides and flavonoids in the leaves of this plant [30], [31].

Table 2 Methods for the identification of secondary metabolites of *Chassalia kolly* leaves

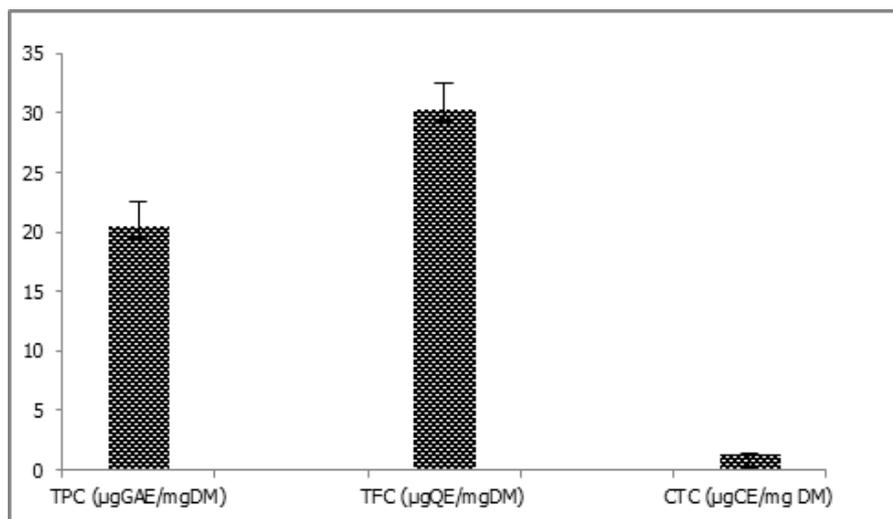
Secondary metabolites	
Alkaloids	+
Tannins	-
Flavonoids	+
Anthocyanins	+
Leuco anthocyanins	-
Reducing compound	+
Mucilage's	+
Coumarins	-
Cyanogenic derivatives	-
Saponosids	+
Anthraquinones	-
Sterols and terpenes	-

Legend: +: present; -: absent

3.2. Phenolic compound content

Figure1 shows the content of phenolic compound (total phenols, total flavonoids and condensed tannins) in the ethanolic extract of the leaves of *Chassalia kolly*.

The contents of total phenols, total flavonoids and total tannins in the ethanolic extract of the leaf of *Chassalia kolly* are respectively $(20.41 \pm 2.11) \mu\text{gEAG}/\text{mg MS}$, $(30.29 \pm 2.18) \mu\text{g EQ}/\text{mgMS}$ et de $(1.211 \pm 0.122) \mu\text{gEC}/\text{mgMS}$. The richness in phenolic compounds, in particular the content of total flavonoids in the leaf of *Chassalia kolly* could explain its use in traditional medicine to treat skin infections [30].

**Figure 1** Phenolic content of ethanolic extract of *Chassalia kolly* leaves

Legends: TPC: Total phenol content, TFC: Total flavonols content; CTC: Condensed tannin content; $\mu\text{gGAE}/\text{mgDM}$: microgram Gallic acid Equivalent per gram of dry matter; $\mu\text{gQE}/\text{mgDM}$: microgram Quercetin Equivalent per milligram of dry matter; $\mu\text{gCE}/\text{mgDM}$: microgram catechin Equivalent per milligram of dry matter

3.3. Antioxidant activity

3.3.1. DPPH free radical scavenging assay

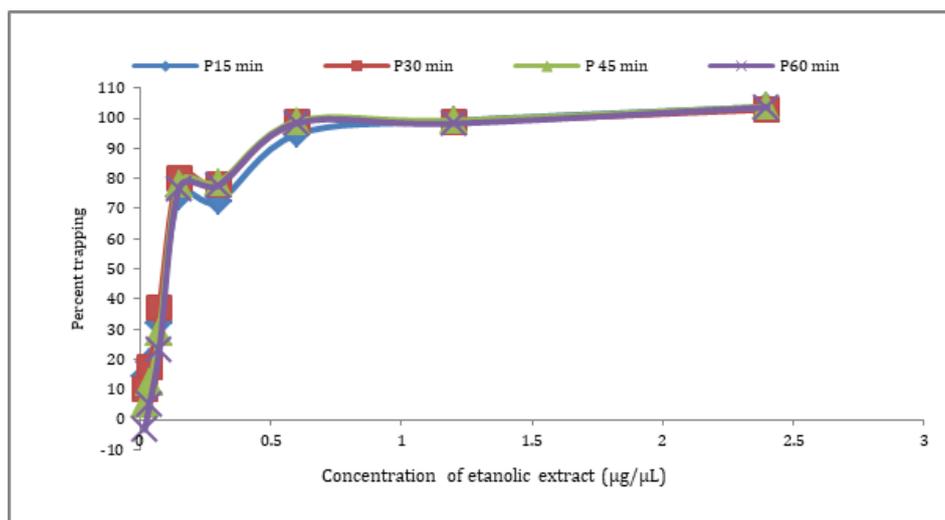


Figure 2 Percentage of DPPH radical scavenging by the ethanolic extract of *Chassalia kolly* leaf

The curves in Figure 2 show the percentage of DPPH radical scavenging as a function of the concentrations of the ethanolic extract from the leaves of *Chassalia kolly*. At the concentrations tested, there is a gradual increase in the percentage of scavenging of the DPPH radical by the ethanolic extract of *Chassalia kolly* leaves up to more than 95% before becoming practically constant. It is noted that the reaction of this extract is simultaneous. After 15 min, the reaction is almost complete. For this extract, the concentration making it possible to trap 50% of the DPPH radical is 0.05 µg/µL. This interesting antioxidant activity of *Chassalia kolly* is linked to the content of phenolic compounds in this plant. This extract could be used to fight against free radical attacks on the skin on the one hand and for the preservation of perishable food products on the other hand instead of these synthetic products.

3.3.2. Total antioxidant capacity of the ethanolic extract of *Chassalia kolly* leaves

The total antioxidant capacity of the ethanolic extract of *Chassalia kolly* is expressed in microgram equivalent of ascorbic acid per milligram of dry matter (µgEAA/mgDM) using the calibration curve plotted with ascorbic acid.

Calibration curve for measuring the total antioxidant capacity

The total antioxidant capacity of the extracts studied is expressed in micrograms equivalents of ascorbic acid per milligram of dry matter from the calibration curve ($y=4.0916x-0.0629$; $R^2=0.9929$) Figure 3. The results obtained are expressed in µg equivalent of ascorbic acid per mg of dry matter (µgEAA/mgDM).

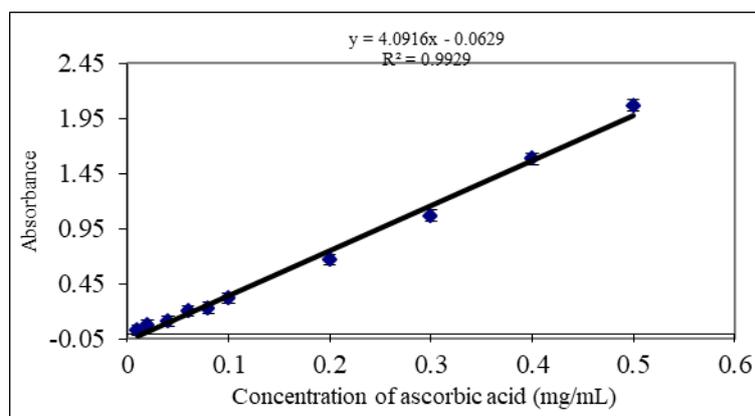


Figure 3 Calibration curve for the evaluation of the total antioxidant capacity

This total antioxidant capacity is $(0,058 \pm 0,002)$ $\mu\text{gEAA}/\text{mgDM}$ for the ethanolic extract of this plant. The interesting activity of this extract is linked to its content of phenolic compounds.

3.3.3. Potential of the extracts to reduce the ABTS⁺ radical

The calibration curve for the determination of the potential of the extracts to reduce the ABTS⁺ radical established with the trolox gives $(y = -0.0057x + 0.3783; R^2 = 0.9989)$; $(y = -0.0051x + 0.337; R^2 = 0.9956)$; $(y = -0.0045x + 0.3086; R^2 = 0.9976)$; $(y = -0.0042x + 0.2872; R^2 = 0.998)$ respectively after 15 min, 30 min, 45 min and 60 min of reaction (figure 2). This potential of the ethanolic extract of *Chassalia kolly* leaf to reduce the ABTS⁺ radical cation as a function of reaction time is expressed in micrograms Equivalence of trolox per milligram of dry matter.

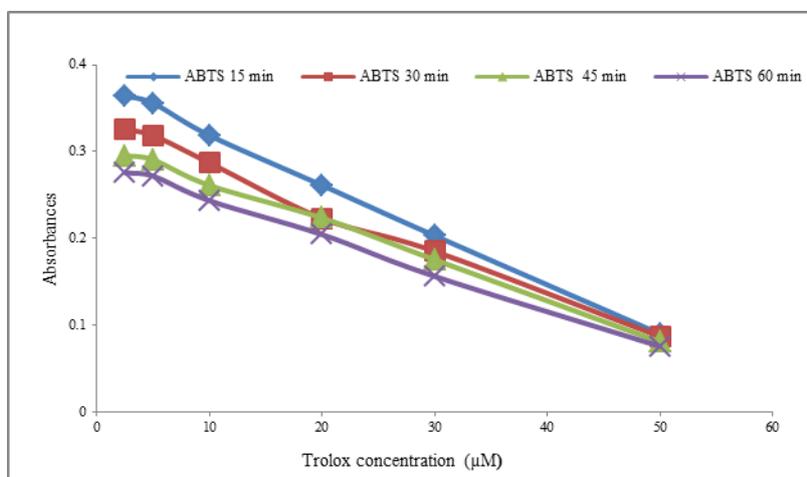


Figure 4 Calibration curve for the potential of the extracts to reduce the ABTS⁺ radical

The potential of the extracts to reduce the ABTS⁺ radical as a function of the reaction time expressed in micrograms Equivalence of trolox per milligram of dry matter is given in Table 3. The reduction potential of ABTS⁺ varies from (33.163 ± 3.255) $\mu\text{gEqTx}/\text{mgMS}$ after 15 min of reaction at (16.908 ± 3.019) $\mu\text{gEqTx}/\text{mgMS}$ after one hour of reaction. Therefore, the potential of the ethanolic extract of *Chassalia kolly* leaf to reduce the radical cation ABTS⁺ gradually decreases over time. From the analysis of this table, it emerges that the ethanolic extract of *Chassalia kolly* leaf showed interesting antioxidant activity. This noted activity would be due to the content of phenolic compounds in this plant. In view of the interesting antioxidant activity of the ethanolic extract *Chassalia kolly*, it could be used to fight against free radical attacks suffered by the skin.

Table 3 Potential of the extracts to reduce the ABTS⁺ radical

Reduction potential of ABTS by the ethanolic extract of <i>Chassalia kolly</i> leaf ($\mu\text{gEq Tx}/\text{DM}$)			
15 min	30 min	45 min	60 min
33.163 ± 3.255	25.841 ± 3.372	20.566 ± 3.514	16.908 ± 3.019

3.4. Anti-inflammatory activity

The percentages of membrane stabilization on red blood cells by ethanolic extract of *Chassalia kolly* leaves and aspirin are given in table 4.

The ethanolic extract of *Chassalia kolly* leaves showed a percentage of limb stabilization of (75.916 ± 0.142) % while for aspirin which is used as a reference compound is (70.125 ± 0.11) %. So the ethanolic extract of *Chassalia kolly* showed more pronounced anti-inflammatory activity than aspirin. This noted activity could be explained by the presence of flavonoids and mucilages in the leaf of *Chassalia kolly* [31], [32]. The interesting anti-inflammatory activity of *Chassalia kolly* could justify its use in traditional medicine to treat skin infections.

Table 4 Red blood cell membrane stabilization potential

Ethanollic extract of <i>Chassalia kolly</i> and reference compound	Percentage of membrane stabilization (%)
Ethanollic extract of <i>Chassalia kolly</i>	75.916±0.142
Aspirin	70.125±0.114

4. Conclusion

Natural substances extracted from vegetal biomass have multiple benefits exploited in industrial biotechnology of food, cosmetics and pharmaceuticals as well. Plant secondary metabolites are a source of potential novel therapeutic. Skin infections still remain the leading cause of morbidity among skin diseases. The present study focused on phytochemical, antioxidant, antimicrobial, and anti-inflammatory properties of *Chassalia kolly* leaves. From the results obtained, it emerges that *Chassalia kolly* leaves have many secondary metabolites including glycosides, alkaloids, flavonoids, anthocynes, reducing compound, mucilages and saponosids.

Regarding antioxidant activity, the ethanollic extract showed interesting activity ($CI_{50}=0.05\mu\text{g}/\mu\text{L}$). The ethanollic extract of *Chassalia kolly* showed more pronounced anti-inflammatory activity ($P=75.916\pm 0.142$) % than aspirin ($P=70.125\pm 0.114$) % which is a reference compound. The results of this study justify the use of *Chassalia kolly* in traditional medicine. Ethanollic extract of *Chassalia kolly* leaves could be used to fight against free radical attacks on the skin, to treat skin conditions and for the preservation of perishable food products instead of using synthetic products to avoid undesirable and toxic side effects on humans being.

Compliance with ethical standards

Acknowledgments

The authors of this manuscript would like to thank the university agency of the Francophony which funded this research project within the framework of the scholarship program "Eugen Ionescu 2019-2020.

Disclosure of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this research paper.

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