***Original Research Article***

 ***Piliostigma thonningii biosafety and efficacy on common actinic damage endpoints***

**Abstract**

*Piliostigma thonningii* from the Fabaceae and Caesalpinioideae sub-family is a huge deciduous tree from the woodlands of Southern Africa that can grow up to 10 meters tall with a rounded crown and a short, often crooked trunk. Prevalent in disturbed areas. *P.thonningii* is medicinally used in African traditional medicine to treat wounds, ulcers, gastric pain, gingivitis and as an antipyretic. The current study systematically, technically evaluated the potential use of the plant in managing photo damage end points through biosafety and pharmacological activity assessments.

Various classical analytical techniques were used to screen for the presence of pharmacological secondary metabolites. The DPPH method was used to determine the anti oxidancy, the egg albumin test was used to determine the anti-inflammatory activity, OECD guideline 426 and 406 were used to determine the acute oral toxicity and the dermal toxicity of the lyophilised extract.

The phytoscreening confirmed the presence of numerous biomedically relevant secondary metabolites including, flavonoids, phenolic compounds, saponins, alkaloids, tannins, phytosterols, proteins and amino acids, carbohydrates, glycosides, terpenoids and lignans. The biosafety study using albino rat models confirmed that *P.thonningii* is nontoxic with an LD50 above 5000mg/kg. The hydroethanolic extract demonstrated high anti-oxidant, high anti-inflammatory and antibacterial activities, which were comparable to the standards, ascorbic acid, diclofenac and ceftriaxone, respectively. The results confirm presence of relevant bioactive secondary metabolites in lyophilized extracts of the leaf parts of *P.thonningii* whichpossess satisfactory anti-inflammatory, antibacterial and anti-oxidant profiles which are all relevant in retarding actinic damage. It was hereby substantiated that the lyophilised extract of *P.thonningii* is biologically active in photo damage retardation and safe thereby justifying its topical use in traditional medicine

**Key words**: *P.thonningii*, anti-inflammatory, antioxidant, antibacterial, actinic damage, albinism, polyphenols, secondary metabolites

# Introduction

##  Piliostigma thonningii

*Piliostigma thonningii* is a deciduous tree species, typically reaching heights of 4–15 m, characterized by a rounded crown and a short, often crooked bole. Twigs are hairy. The twigs exhibit pubescence, while the bark is rough, longitudinally fissured, and transitions from creamy-brown when fresh to grey brown upon aging. The leaves are leathery, green, and broadly bilobed (up to 15 × 17 cm), with lobes extending one-eighth to one-third of the leaf length and a small bristle present in the apical notch. The adaxial surface is glossy, while the abaxial surface is heavily veined and covered with rusty-brown trichomes (Figure 1). The seeds are encased in an edible pulp1. This species is commonly found in open woodlands, wooded grasslands, and gallery forests, predominantly at low to medium altitudes. It exhibits broad edaphic tolerance but shows a preference for heavy clayey or medium loamy soils. *P. thonningii* is a pioneer species, frequently persisting in cultivated fields and rapidly colonizing disturbed areas 2. The bark is widely utilized for its fibrous properties, serving as a binding material in hut construction, fencing, and bridge-building. In Sudan and Mozambique, the inner bark is traditionally used to secure arrowheads to shafts. Leaves function as food wrappers, while twigs and roots are employed as oral hygiene implements (chewing sticks). The dried fruit is used in pottery polishing, and root extracts provide a glaze for gourds3. *P.thonningii* is used medicinally in many African countries to treat wounds, ulcers, gingivitis and as an antipyretic 2. It also has significant non-medicinal applications in livelihood serving as fodder, shade, food, and wood. It also improves the productivity of farm products by improving soil fertility and provides a nutritional feed that improves the survival and productivity of domestic animals 4. Over the years, there have been various reports on the bioactivity of the different parts of the plant that revealed interesting pharmacological activity such as antimicrobial, free radical chelating (antioxidant), and anti-inflammatory4. *P. thonningii* holds significant ethnopharmacological value across Africa. Root preparations are applied as haemostatics for wound and ulcer management, as well as remedies for helminthiasis, diarrhoea, dysentery, and cough. Additionally, they serve as diuretics and antidotes for snakebites. Leaf decoctions are used topically to treat fractures and bone inflammation, while steam baths derived from boiled leaves alleviate musculoskeletal inflammation. In southern Africa, leaf infusions regulate menorrhagia and act as antiemetics. Ophthalmic treatments incorporate leaf sap, and in Côte d’Ivoire, powdered flower buds and young leaves are administered orally to mitigate palpitations. Senegalese traditional medicine employs the leaves in managing dementia5.

Beyond its medicinal value, *P. thonningii* enhances agro ecosystems by improving soil fertility and providing high-nutrition fodder, which boosts livestock productivity. It also serves as a shade tree and source of fuel wood, underscoring its multifunctional role in rural livelihood4.



Figure 1: P.thonningii aerial parts and pods

## Secondary metabolites and photo damage

Photo protection can be defined as a group of mechanisms designed to lessen exposure to sunlight. It serves as the primary defense against skin conditions brought on by ultraviolet radiation. These measures include oral photo protectors and photo protective ingredients in topical sunscreens. The molecules' ability to absorb, reflect, or scatter sun radiation determines the topical photo protective capacity. Although they cannot directly shield the skin, oral photo protectors can strengthen or activate the skin's defenses 6. Compounds with aromatic rings, specifically phenolic compounds and flavonoids, have a great capacity to absorb both UVA and UVB rays 7,8.

Phenolic compounds, there just about 800 different structures of phenolics known 9. A common classification of phenolic metabolites distinguishes the flavonoid and non-flavonoid compounds, flavonoids having two aromatic rings connected by a bridge consisting of three carbons and non-flavonoids having the following subgroups: phenolic acids, lignans and stilbenes 10. The health-promoting effects of phenolic compounds on the skin are photo protection, anti-inflammatory, antiaging and photo-chemoprevention. The presence of these properties is due to the structure of the phenolic compounds that is the phenolic rings and hydroxyl groups that produce a potent free radical scavenging and antioxidant activity 11. Phenolic compounds antioxidant activity can be explained by the following mechanisms of action: (i) inhibiting ROS biosynthesis; (ii) trapping ROS; (iii) reduce metal ions catalysts of ROS synthesis 9. Phenolics anti-inflammatory mechanisms are not fully known, but reports have stated that phenolic compounds employ this effect by: (i)neutralizing free radicals such as ROS and reactive nitrogen species (RNS); (ii) inhibiting activated immune cells, lipid peroxidation and pro-inflammatory mediators, such as interleukin 6 (IL-6) and prostaglandin-E2 (PGE2); (iii) modulating transcriptional factors, such as nuclear factorkB (NF- kB) or Nrf-2, in inflammatory and antioxidant pathways; (iv) modifying eicosanoid synthesis 12,13.

Non-flavonoid phenolics reported to have skin photo protective activity are: vanillic acid, p-coumaric acid, caffeic acid, ferulic acid, rosmarinic acid, chlorogenic acid, gallic acid, tannic acid, as well as resveratrol and curcumin 11. The two phenolics with the greatest research on photo protection are caffeine and ferulic acid. By preventing the lipid peroxidative chain reaction, these two phenolic compounds have been shown to shield phospholipidic membranes against UVR-induced peroxidation. By controlling antioxidant defenses such glutathione, catalase, and glutathione peroxidase, both substances offered photo protective benefits against oxidative stress, MMP-1 activation, and ROS production in human keratinocytes14. According to a recent study, these two significant PCs may have an indirect regulatory influence on the nuclear factor erythroid 2-like 2 pathway in melanoma cells, protecting against UVA-induced melanogenesis 15.

Flavonoids are one of the major classes of phenolic compounds contributing to photo protection. These molecules absorb UVR and reduce ROS oxidative damage. The double bonds present in the flavonoid structure give them a high capacity to absorb UVR, and the hydroxyl groups attached to aromatic rings contribute to their ROS scavenging capacity9

Carotenoids, these are tetraterpenoids which have a central carbon chain with alternating single and double bonds carrying different cyclic or acyclic end group16. They have photo protective qualities due to their extended system of conjugated double bonds, which also allows them to absorb UVR, inhibit lipid peroxidation, and physically quench radicals like peroxide and singlet molecular oxygen produced during photooxidation17. Additionally, because carotenoids can activate phase 2 cytoprotective genes, they can trigger cellular defenses. Stahl and Sies explore the processes by which dietary carotenoids provide photo protection 18.

Alkaloids, there are a group of compounds identified by the presence of a nitrogen atom in a heterocyclic ring19. Caffeine, theophylline, and theobromine have all been examined for their photo protective and antioxidant qualities. Caffeine is the alkaloid that has been studied the most in relation to photo protection 20,21. Caffeine has been shown to have anticancer effects when applied topically and taken orally. Caffeine used topically decreased skin carcinogenesis in mice exposed to UVR radiation. Caffeine used orally reduced the volume, multiplicity, and incidence of tumours22. Additionally, it enhanced apoptosis in mice's UVB-induced skin cancers in a selective manner23. Increased caffeine use is linked to a lower incidence of skin cancer, especially basal cell carcinoma (BCC), according to epidemiological research. The rationale behind this impact is that cells with damaged DNA undergo an increase in apoptosis, while surprisingly, normal epidermal cells do not11. Several compounds present in plants have been shown to have positive effects on skin health as well as photo protective properties11.

## Antioxidants and photo-damage

The most common component thought to irritate human skin cells is ultraviolet light. There are two forms of UV radiation that reach the earth's surface: short wavelength UV-B and UV-A, which makes up the majority of the spectrum 24. Melanin production, endogenous antioxidant defense, and antioxidant chemicals that are either ingested or applied topically are all involved in protecting the human skin. DNA undergoes photochemical changes, including pyrimidine dimers, when exposed to UV light 25,26. When exposed to ultraviolet light, oxidative stress is produced. The human skin is thought to withstand 105 oxidative damage events every day. Nevertheless, given the quantity of oxidative events, the cancer induction rate is far lower than anticipated because the DNA is rather durable27. As we age, the skin's ability to defend itself against free radicals dramatically declines. The availability of antioxidant molecules from exogenous sources, such as diet or cosmetics, is constantly required to replenish the skin's antioxidant defensive qualities28.

Based on their strong antioxidant qualities and SPF assay results, the phenolic chemicals found in the extract (ethyl gallate, gallic acid, and a combination of flavonoids) were deemed promising natural photo protectants29.

## Anti-inflammatory role in actinic damage treatments

Inflammation is a significant component of photo induced oxidative damage. Numerous data on the attenuation of inflammatory mediators by phenolic compound activity may be found in the literature. The signaling molecules prostaglandin-E2 (PGE2) and interleukin 6 (IL-6) are often cited 9. Topical 3% diclofenac in 2.5% hyaluronic acid gel (Solaraze) is currently the only NSAID licensed for the treatment of actinic keratoses30, containing an anti-inflammatory agent for treatment of photo damage.

# Materials and methods

## Materials, equipment and facilities

All chemicals, associated reagents, equipment and facilities for the *in-vivo* laboratory animal toxicity investigations and the bioactivity assays were obtained from the University of Zimbabwe, Faculty of Medicine and Health Sciences laboratories, Pharmacy and Pharmaceutical Science Department.

### Animal use approval

Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC) which is the local research Institutional Review board for the University of Zimbabwe.

### P Thonningii Plant material collection and preparation

Fresh leaves of *P.thonningii* were collected in Dema, Seke District Mashonaland East, Zimbabwe, (18.0833° S, 31.2167° E) and were taken for authentication at the National herbarium of Zimbabwe where they were approved. The leaves were washed and air dried in the shade for 1 week. The dried samples were ground into small fragments. The coarsely ground leaves were crushed into a fine powder using a Thomas-Wiley laboratory mill model.

400g of the obtained powder was soaked into 70% ethanol (1400ml 100% ethanol in 600ml distilled water) for 5 days. The solution was filtered first using a mutton cloth to remove the coarse particles and then the Buchner filter with the desired product being the filtrate. The rotary evaporator was used to eliminate the ethanol from the filtrate leaving the liquid extract. The liquid extract was freeze dried and the measure mass of the powdered extract was obtained.

## Phytochemical Screening of Piliostigma thonningii

In a 200ml round bottomed flask, 10g of the lyophilized hydro-ethanolic extracts of *P.thonningii*  were dissolved in 100ml of distilled water and subjected to various phyto-screening techniques to confirm the presence or absence of relevant phytoconstituents of pharmacological interest. The following qualitative tests were conducted on the extract liquor.

### Detection for alkaloids by the Iodine test

The Iodine test was used to determine the presence of alkaloids. In this assay, to 3ml of the lyophilized extract solution, a few drops of iodine solution were slowly added along the sides of the test tube. The presence of alkaloids was then identified by the appearance of a blue colour, which disappears on boiling and reappears on cooling 31.

### Detection of tannins by the Braymer’s test

The simplified Braymer’s test was used to detect the presence of tannins. To 1ml lyophilised extract solution, 3 drops of a 10% Ferric chloride solution were added. The presence of tannins was confirmed by conversion of the solution to a blue-green colour32.

### Detection of flavonoids by the Ammonia test

Flavonoids were detected by means of the Ammonia test where 5ml dilute ammonia solution was added to 5ml of the lyophilised solution followed by a few drops of conc. H2SO4. The emergence of a yellow colour indicates the presence of flavonoids33.

### Detection of Glycosides by the Keller-Killani test

The presence of glycosides was done by the Keller-Killani test34. To 1mL of the lyophilised solution, 1.5mL glacial acetic acid was added and a few drops of 5% ferric chloride were added as well as conc. H2SO4 (along the side of test tube). The presence of glycosides was confirmed by the emergence of a blue coloured solution in mixture acetic acid layer.

### Detection of Phenolic compounds by the Ferric chloride test

The presence of phenolic compounds was done by the ferric chloride test. To 1ml of lyophilised extract solution 2 to 3 drops of FeCl₃. The presence of phenolic compounds was observed by a blue-green colour 35.

### Detection of saponins by the simplified foam test

The simplified foam test was used to determine the presence of saponins. In this assay 2ml of the extract was added to 20ml distilled water. The mixture was shaken in a graduated cylinder for 15 minutes. The presence of saponins would be confirmed by the formation of form with a head height of at least 1cm36.

### Quantification of Total Phenolic and Tannins content

The total phenolic and tannins content of the lyophilised *P.thonningii* were determined according to an amended Folin-Ciocalteu spectrophotometric method . To prepare a calibration curve, phenol (Gallic acid) stock solution (5 mg/mL) was added into 100mL volumetric flasks, and then diluted to volume with distilled water. From each calibration solution, 0.25 mL was mixed with 1.25 mL of 10-fold diluted Folin-Ciocalteu’s phenol (1 mL Folin reagent and 9 mL deionized water) reagent and allowed to react for 5 minutes. Then, 2 mL of 7.5% Na2CO3 solution was added, and the final volume was made up to 5 mL with deionized water. After 1 hour of reaction at room temperature, the absorbance at 760 nm was determined spectrophotometrically (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments). The test was done in triplicate. Calibration curves were plotted to determine the level of phenolics and tannins in the samples. The total phenolic contents were calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of the sample (comparison was made between ethanolic and methanolic extracts in this assay).

### Quantification of total Flavonoids

The total flavonoid content of the lyophilised *P.thonningii* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water. To this solution, 0.5mL of 1M sodium nitrite was added together with 2ml of a 1M, NaOH solution. Distilled water was then added to make up to 10ml volume. The solution was shaken and allowed to stand at room temperature for 15 min and the absorbance was subsequently measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve37.

## Anti-oxidancy evaluation of *Piliostigma thonningii* using the DPPH free radical scavenging assay

The antioxidant activity of the lyophilised *P.thonningii* hydroethanolic extracts was determined using the DPPH free radical scavenging assay38. Carefully measured 50 µL of the lyophilised plant extract solution in concentrations ranging from 5 to 150 mg/ml was added to identical bottles, and to each 5 ml of a 0.004% (w/v) solution of DPPH was added. The resultant mixture was agitated and incubated at 22oC (Shel lab SRI3 Low Temperature BOD Incubator) in darkness for 30 minutes. Discoloration was measured and readings taken in triplicate at 517 nm using a UV spectrophotometer (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments). The blank was 70% (v/v) methanol. Ascorbic acid was used as a reference standard. The DPPH scavenging effect was calculated using the following equation,

***Equation 1***

$$DPPH Svavenging percentage effect= \frac{A^{o}-A}{A^{o}} x 100$$

Where Ao is the absorbance of negative control (0.004% DPPH solution), and A is the absorbance in the presence of extract.

The results were reported as IC50 values and ascorbic acid equivalents (AAE, mg/g) of *P.thonningii.*

## Anti-inflammatory activity of *Piliostigma thonningii* using the egg albumin denaturation test

The anti-inflammatory activity of the lyophilised leaf extract of *P.thonningii* was determined using the egg albumin protein denaturation assay, with slight modifications as described by Chifamba *et a.l* (2024)39. The samples and reagents used for this assay include 0.4 mL of egg albumin (fresh) from a free-range domesticated hen (*Gallus domesticus*), 10 mL of phosphate-buffered saline (PBS) at pH 7.2, and 5ml solutions of varying concentrations of the lyophilised leaf extracts in 0.4% DMSO. The concentrations of the lyophilized extracts in the total reaction solution ranged from 50 to 1000 µg/mL. The samples were incubated (Shel lab SRI3 Low Temperature BOD Incubator) for 20 minutes at 37°C; by heating at 65°C in a water bath for an additional 30 minutes to induce denaturation of the egg albumin. After cooling the mixture, the absorbance was measured at 660 nm (UV spectrophotometer, Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments) using the vehicle as blank. Negative controls consisting of 0.4 mL of fresh egg albumin, 0.5 mL of 0.4% DMSO, and 3 mL of PBS were included in the experiment. Diclofenac sodium was used as a positive control for the study at similar concentrations. The percentage of inhibition, which translates to the anti-inflammatory activity of the extracts and standards, was calculated by the following equation

***Equation 2***

$$Inflamation inhibition percentage effect=\frac{Abs\_{sample}}{Abs\_{control}-1} x 100$$

Where,

Abs sample = absorbance of sample, Abs control = absorbance of control.

## Antibacterial evaluation of *Piliostigma thonningii*

### Test microorganisms

The bacterial species; *Staphylococcus Aureus* a gram-positive bacterium as well as gram negative *Escherichia coli* were obtained from the University of Zimbabwe Department of medical microbiology in Harare, Zimbabwe. The antibacterial, evaluation laboratory studies were performed according to CLSI guidelines.

### Determination of the zone of inhibition

A modified, simplified Kirby-Bauer testwas configured and used in this study to determine antibacterial properties of crude *P.thonningii* through zone of inhibition observations and MIC determination through serial dilution techniques 4.

### *Zone of inhibition MIC test requirements*

* Mueller-Hinton agar plates
* Sterile swabs and forceps
* Pure bacterial cultures
* *P.thonningii* extract liquor

### Zone of inhibition measurement and MIC determination test protocol

Bacterial inoculum suspensions were spread uniformly on solidified Muller−Hinton Agar (MHA) using sterile swab. The bacterial strains used in the study were *Staphylococcus aureus* as well as gram negative *Escherichia coli*.

In the procedure: To the Mueller-Hinton agar plates, swabs of the pure bacterial cultures were evenly spread over and 2 -3 drops of the test samples were placed in the media plate using sterile forceps.  The petri plates were incubated for 24 hours at 36oC with controlled humidity. After the incubation period and diffusion of the test samples, the clear area (zone of inhibition) around the point of introduction of the samples was observed and measured. The size of the zone of inhibition is directly proportional to the antibacterial activity.

For the determination of MICs the same experimental set up was used. Serial dilutions of the test materials with distilled water was done and the minimum concentrations of the test materials needed to inhibit the ability of the microorganism’s ability to produce any visible growth in the agar plates was noted. In this simplified modified method, the lowest concentration of the antimicrobial agents (in µg/ml) which prevented the appearance of visible growth of the microorganisms within a 24 hour period were determined as the MIC.

## Acute oral and dermal toxicity evaluation of *Piliostigma thonningii*

The acute oral toxicity evaluation of *P.thonningii* lyophilized extract was done using a modified OECD technical guideline 425 (The up and down test)40. Female nulliparous Wistar rats (24) were used, which were acclimatized to the test environment for 10days prior to the commencement of the test protocols. The participating animals were fed with a commercial standardized rodent pellet from Agrofeeds® and were given water *ad libitum*. The animal habitat was kept at an average ambient temperature of 25°C throughout the study with a relative humidity level of 40% and an artificially controlled photoperiod of 12-h light and 12-h darkness. The animal welfare, observations and care were supervised by a practicing veterinary officer.

In our test, sequential ordered progressions of doses were orally administered to the animals at 48-hour intervals. The animals were divided into 2 groups of 12 female rats each; the first group (group 1) received distilled water and served as the control group. The second (group 2) received incremental doses of the *P.thonningii* solution. The selected animals were marked so as to facilitate individual identification. . The experimental animals were fasted for 18 hours with water prior to dosing. Initial starting doses were chosen based on related toxicological studies. The first animal received a dose of 250mg/kg body weight, which was below a randomly selected estimated LD50. When animals survived the dose, the next dose was doubled, subject to our observations of the test animals over a period of 48 hours. The *P.thonningii* was orally gavaged in a water solution in 4 different sets of doses of: 250, 500, 1000, 2500 and 5000 mg/kg body weight. The female rats were observed by a veterinary specialist for morbidity and mortality twice daily. In the absence of mortality, the rats were observed for any visible changes and clinical signs and symptoms of toxicity every 1 hour, and up to 12 hours on day 1, and thereafter, once daily up to a maximum of 14 days. The animals were also weighed daily.

The skin sensitivity tests conducted on the *P.thonningi* ethanolic extracts were guided by OECD technical guideline 406 with minor amendments using 3 adult male New England breed white laboratory rabbits, weighing between 1.3-1.8kgs 41. The rabbits were checked for their suitability for the study over a 7-day acclimatization period. The rabbits were kept in a rodent facility in a limited access facility. They fed on a typical commercial rabbit diet and had unlimited access to drinking water. Prior to the test, the backs of the rabbits were shaved by depilatories, and the shaved area was divided into two marked parts measuring 25cm2 each. The first marked area was used for the application of the extract and the second demarcated area was used as the control for testing the irritation according to OECD guidelines41.

In our test, 2ml of the ethanolic *P.thonningii* extract was applied by a syringe and spread evenly over the 25cm2 demarcated shaved area of each animal. The application site was covered by gauze and the area was lightly covered by non-sticky bandages. The treated rabbits were then returned to their respective cages and observations were made on the sites at 24, 48 and 72 hours. Any sensitivity or reactions to the treatment was evaluated by the following criteria (Table 1) as per the documented method 42.

Table 1 Observed irritation classification

|  |  |  |
| --- | --- | --- |
| Reaction | Observation | Score |
| Erythema | No erythema | 0 |
|  | Very slight erythema | 1 |
|  | Well-defined erythema | 2 |
|  | Moderate to severe erythema | 3 |
|  | Severe erythema to eschar formation | 4 |
| Oedema | No oedema | 0 |
|  | Very slight oedema | 1 |
|  | Well defined oedema | 2 |
|  | Moderate oedema (raising 1mm) | 3 |
|  | Severe oedema (raised more than 1 mm and extending beyond area of exposure) | 4 |

# Results and discussion

## Phytochemical screening of Piliostigma thonningii

From the phytochemical screening protocols, our investigations confirmed the abundance of primary and secondary metabolites of biomedical relevance with regards to actinic damage (Table 2). The compounds with the strongest presence in the plant were phenolics (especially flavonoids), tannins, saponins, alkaloids. These results correlate with studies by Ighodaro et al 4, who identified tannins, flavonoids, alkaloids, and saponins among other compounds in *P.thonningi*. This proliferation of medically relevant phytoconstituents validates the numerous uses of the plant in traditional medical practice in general, and the management of actinic damage in particular. These phytochemicals exhibit significant photoprotective effects against ultraviolet radiation (UVR)-induced skin damage through multiple mechanisms, including UV absorption, antioxidant activity, melanin regulation, and anti-inflammatory action. Studies in both in vitro and in vivo models have demonstrated that antioxidant phytochemicals mitigate photodamage by directly scavenging reactive oxygen species (ROS), enhancing endogenous antioxidant defenses, and modulating key signalling pathways associated with inflammation, DNA repair, and skin cell viability and function42, 43.

Table 2 : Qualitative screening of P.thonningii secondary metabolites

|  |  |  |
| --- | --- | --- |
| Test | Presence in hydro-ethanolic extract | Presence in distilled water extract |
| Alkaloids | ++ | + |
| Phytosterols | +++ | ++ |
| Flavonoids | +++ | + |
| Saponins | +++ | ++ |
| Proteins and Amino Acids | ++ | + |
| Fixed oils and fats | - | - |
| Phenolic compounds | +++ | + |
| Tannins | ++ | + |
| Carbohydrates | ++ | + |
| Glycosides | ++ | + |
| Terpenoids | +++ | + |
| Lignans | +++ | + |

*(-): Indicates the absence of the phytochemical*

 *(+): Indicates the presence of the phytochemical*

*(++): Indicates moderate presence of the phytochemical*

*(+++): Indicates strong presence of the phytochemical*

### Total phenolic content

All the extracts from the 2 different solvents (ethanol, and water), had considerable total phenolic content (TPC). The results of the TPC of the hydro-ethanolic extract was higher than both the hydroethanolic and aqueous extracts (Figure 2). This result can be explained by the fact that ethanol has a higher London dispersion force than methanol due to its higher molecular weight which leads to stronger intermolecular attraction than methanol.

Figure 2 Total phenolic content for P.thonningii extract

The TPC represent the largest, diverse group of related secondary metabolites produced by plants which share a related sub structure composed of an aromatic-OH moiety43. Phenolics are produced mainly as a defence mechanism in response to hostile environmental biotic or abiotic stresses. Plant phenolics are mostly hydroxybenzoic and hydroxycinnamic acid derivatives. There has been increased interest towards their medicinal use as antioxidant, photoprotective, anti-inflammatory and wound healing activities. Plant phenolics are the main group of secondary metabolites that function as antioxidants or reactive species scavengers. To this regard, these plant polyphenols mostly operate as singlet oxygen scavengers as well as reducing agents. In our study we preferred the Folin-Ciocalteu method compared to the others. In the present study the results were observed through the formation of a blue-coloured solution after the addition of the lyophilised extracts to the Folin-Ciocalteau reagent in the conducive medium. The colour change was due to the *in-situ* formation of the phospho molybdic-phosphotungstic-phenol complex. The TPC was calculated aided by the use of the regression equation of the calibration curve (R2 =0.989, y = 0.009x + 0.0464), expressed in GAE as milligrams per gram of the extract or fraction (mg GAE/g extract or fraction). As shown in Figure 3 and Figure 4, the 2 extracts yielded phenolic compounds in the following order: hydroethanolic > distilled water.

Figure 3: Total Flavonoid content for P.thonningii extracts

Flavonoids are a class of polyphenolic compounds that are differentiated by the chemical structures yielding subgroups such as flavones, flavonols, flavanones and flavanols. Flavonoids possess potent antioxidant activities44 (Figure3). These contribute to the scavenging of 2, 2-diphenyl-1- picrylhydrazyl (DPPH) as shown in this study. Other related studies have demonstrated their affectivity against super oxides and ABTS radicals; and strong iron chelating activity. The most abundant flavonoids isolated from the aerial parts of *P.thonningii* in published studies include rutin, kaempferol and quercetin. These flavonoids have displayed anti-inflammatory, arterial blood pressure and anti-diuretic activities in other studies.

## Antioxidant Assay of Piliostigma thonningii

DPPH scavenging activity was 93.4% for ascorbic acid (the standard used) at 150 µm/ml. While *P.thonningii* hydro-ethanolic extract was 78.8% and 57.5% for the aqueous extract. Activity was determined from the absorbance obtained at specific concentrations (Figure 3). The IC50 values were 26.75µg/ml, 60.25µg/ml and 100.01µg/ml for ascorbic acid, *P.thonningii* hydro-ethanolic extract and the aqueous extract respectively. Antioxidants exert their free radical scavenging activities via diverse mechanisms including reducing power, chelating transition metal, radical scavenging activities, and disintegrating radicals. In our studies we used the DPPH radical. The DPPH is a methanol soluble compound characterized by a deep-violet colour that exhibits maximum UVR absorption at 515 nm. The test principles are based on the fact that reactive species are able to reduce DPPH to 2,2-diphenyl-1-hydrazine (DPPH-H) or a substituted analogous hydrazine (DPPH-R) characterized by colourless or pale-yellow colour46. This colour change can easily be tracked spectrophotometrically. Previous scholars established that the participating metabolites in antioxidant activities of *P.thonningii* include polyphenols, lignans, phytosterols, as well as enzymes. These metabolites are able to inhibit the oxidation related colour change. In our studies, although both aqueous and hydro-ethanolic extracts showed good inhibitory performance with respect to the DPPH radical, the hydro-ethanolic extract was more superior. The % inhibition was directly proportional to the extract concentration as shown in (Figure 4 and 5).

Figure 4: Absorbance vs. concentration of P.thonningii

Figure 5: Antioxidancy activity of P.thonningii

We can therefore confirm that that *P.thonningii* extracts are rich in various antioxidant compounds, which help mitigate oxidative stress, a contributing factor in actinic damage preventions and treatments 4. Although not investigated in our study, photoprotective properties of *P.thonningii* are well documented.

## Anti-inflammatory tests of Piliostigma thonningii

Table 3: Anti- Inflammatory activity of lyophilised P.thonningii extracts.

|  |  |  |
| --- | --- | --- |
| Concentration | % Inhibition | % Inhibition |
| µg/ml | ***P.thonningii* hydroethanolic extract** | **Diclofenac (standard)** |
| 250 | 15±0.8 | 25.4±1.2 |
| 500 | 28.6±1.1 | 38.7±1.5 |
| 1000 | 42.3±1.5 | 55.2±1.8 |
| 2000 | 58.7±2.0 | 72.4±2.2 |
| 4000 | 71.5±2.3 | 85.1±2.5 |
| 6000 | 79.8±2.6 | 90.3±2.7 |
| 8000 | 84.2±2.8 | 93.6±2.9 |

At related concentrations, the lyophilised extracts from the aerial parts of *P.thonningii* exhibited anti-inflammatory effects that are approximately a third of the anti-inflammatory attributes of the standard Diclofenac (Table 3). The anti-inflammatory activity of *P.thonningii* became comparable to the least dose of the positive control Diclofenac (250 µg/ml) used in this study at approximately around 750µg/ml. The anti-inflammatory effect of 2000 µg/ml of the positive control Diclofenac was approximately 6% greater than that for 8000 µg/ml lyophilised crude extracts of *P.thonningii*. Much higher values were administered in previous studies using the carrageen induced oedema foot model, where the root extract of *P.thonningii*  inhibited acute oedema in a dose dependent manner from 30-300mg/kg, with a maximal inhibition of 53.8±8.2% attained over 4 h period. Although different methods are used here to represent inhibition of inflammation, the value of 8000µg/ml recorded in our protein denaturation assay, represents significant anti-inflammatory potential of *P.thonningii*, since the extract was used in its crude form. Activity is expected to increase further using bioactive fractions and isolated compounds. Phytosterols, triterpenoids such as lupeol and oleanolic acid, sesquiterpenes, and flavonoids such as rutin and kaempferol have been associated with anti-inflammatory activity of the *P.thonningii*4. Saponins which are normally found in abundance in the plant, have also shown anti-inflammatory activity in addition to anti-oxidant capacity. However, our findings suggest that by targeting chronic inflammation pathways, *P.thonningii* can potentially improve skin defences for photo-protection, as well as management and prevention of actinic damage in albinistic skin types.

## Antibacterial tests on *P.thonningii*



Figure 6: antibacterial activity of water, ceftriaxone and P.thonningii against E.coli was observed



Figure 7: antibacterial activity of water, ceftriaxone and P.thonningii against Staphylococcus aureus was observed

*P.thonningii* ethanolic extracts presented with moderate antibacterial activity, against ceftriaxone a positive control( Figure 6 and 7). The extract showed little antibacterial activity with *E.coli*, but moderate activity with *staphylococcus aureus*. Phytochemical compounds such as alkaloids, saponins, tannins, flavonoids and steroids which are also present in *P.thonningii* have been said to be biologically active and thus partially responsible for the antimicrobial activities of plants, thereby explaining the antibacterial activity of *P.thonningii*45. These results correlate with a study by Ighodaro showing *P.thonningii* with moderate antibacterial effects.

## Acute oral toxicity and dermal evaluation of hydroethanolic extracts *Piliostigma thonningii*

### Acute oral toxicity evaluation of hydroethanolic extracts of P.thonningii

Table 4: Acute oral toxicity study of P.thonningi behavioural observations

|  |  |
| --- | --- |
| Observed parameter |  Dose of *P.thonningii* in mg/kg body weight |
|  | **250mg** | **500mg** | **1000mg** | **2500mg** | **5000mg** | **Control** |
| Food intake | Normal | Normal | Normal | Normal | Normal | Normal |
| Water intake | Normal | Normal | Normal | Normal | Normal | Normal |
| Death | Alive | Alive | Alive | Alive | Alive | Alive |
| Breathing | Normal | Normal | Normal | Normal | Normal | Normal |
| Diarrhoea | Not observed | Not observed | Not observed | Not observed | Not observed | Not observed |
| Urination | Normal | Normal | Normal | Normal | Normal | Normal |
| Skin colour | Normal | Normal | Normal | Normal | Normal | Normal |
| Drowsiness | Not observed | Not observed | Not observed | Not observed | Not observed | Not observed |
| Erection of Fur | Not observed | Not observed | Not observed | Not observed | Not observed | Not observed |

The acute toxicity study was carried out as per OECD technical guideline 425. The observations, results and interpretation were done by a qualified veterinary expert. Our findings indicated that the extract at doses up to 5000 mg/kg body weight imparted neither visible signs of toxicity nor mortality in rats, suggesting its safety. No animals were withdrawn from the study for any reason during the observation period(Table 4). Our results are in agreement with studies by Ukwuani AN et.al46, where the LD50 of the *P.thonningii* extract was estimated to be > 5000 mg/kg body weight. Our extracts were deemed non-toxic, based on the toxicity classiﬁcation proposed by Loomis and Hayes47, which categorise substances with LD50 values from 500 to 5000 mg/kg as slightly toxic and those with LD50 values 5000 to 15,000 mg/kg body weight are regarded as practically non-toxic. This therefore implies that the use of high extract concentrations to achieve the desired bioactivity effects will not pose any toxicity in *P.thonningii* based treatments.

## Rat weights observations

Figure 8: Observed rat weights during acute oral toxicity studies of P.thonningii

In toxicity evaluations, generally, unexpected fluctuations in body weight are a simple and sensitive reflection of toxicity after exposure of study animals to materials. Progressive Weight loss or gain of animals is usually indicative of stress, failure to feed or a response to observed or underlying adverse health conditions. In the present study, the lyophilised extracts did not signiﬁcantly affect normal body weight growth during the study period suggesting that the extract did not alter rat growth at the concentrations investigated (Figure 8).

### Acute dermal toxicity

Table 5 Erythema and oedema scores for rabbit skin sensitivity

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | *P.thonningii* ethanolic extracts | Control(water) |
| Rabbit | **Skin sensitivity reactions** | **24hours** | **48hours** | **72hours** | **24hours** | **48hours** | **72 hours** |
| 1 | Erythema | 0 | 0 | 0 | 0 | 0 | 0 |
|  | Oedema | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | Erythema | 1 | 0 | 0 | 0 | 0 | 0 |
|  | Oedema | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Erythema | 1 | 0 | 0 | 0 | 0 | 0 |
|  | Oedema | 0 | 0 | 0 | 0 | 0 | 0 |

The skin irritation and sensitivity test on rabbits for the ethanolic extract and water exhibited the following: Erythema: After 24 hours, the skin irritation score for erythema and oedema in all 3 rabbits ranged from 0 to 1 for both the ethanolic extract and water. No erythema or oedema values above 1 were observed on any rabbit in all the studies carried out (Table 5). Recorded scores of 0 after 72 hours, showing disappearance of the slight erythema within a short tie fame. Oedema: For the oedema, the results for the extract and water were also identical. No animal exhibited any signs of oedema formation after application of extracts and water. After the 72 hours, time frame, no rabbit was exhibiting any form of erythema or oedema, and the experiments were concluded as per the guidelines.

# Conclusions

The aerial lyophilised hydro-ethanolic extracts of *P.thonningii* were shown topossess considerable antioxidant, anti-inflammatory and antibacterial activities. The observed activities were attributable to the presence of secondary metabolites including lignins, tannins, alkaloids, phenolic compounds, flavonoids, and phytosterols. These contribute to the underlying mechanisms behind the plant’s proven photoprotective effects and therapeutic activities in actinic damage. *P.thonningi* was nontoxic at 5000mg/kg and was seen to have no dermal irritation. Our biosafety and bioactivity studies therefore authenticate the use of *P.thonningii* as a potential actinic damage in albinistic skin types remedy in traditional medicine.

# Disclaimer (Artificial intelligence)

The Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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