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Comparative wound healing potential of *Mitracarpus hirtus* ointment and honey in diabetic albino rats by collagen assessment

BAWA INALEGWU, JACOB AONDONGUSHA JATO♥, OVYE AKYENGO, JOHN AKIGHIR
Department of Biochemistry, University of Agriculture Makurdi. P.M.B 2373, 970001 Makurdi, Nigeria.
Tel.: +234-8097037099, ♥email: jatojack@gmail.com

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Abstract. *Inalegwu B, Jato JA, Akyengo O, Akighir J. 2021. Comparative wound healing potential of* Mitracarpus hirtus *ointment and honey in diabetic albino rats by collagen assessment. Biofarmasi J Nat Prod Biochem 19: 39-44.* All humans will experience some type of wound in every lifetime. Most wounds heal quickly with little or no attention, but many people suffer from complex and/or persistent wounds, therefore posing a burden. This study was designed to assess the efficacy of *Mitracarpus hirtus* (L.) DC. ointment against honey in diabetic rats. To achieve this, percentage wound closure and collagen assessments were used to express treatment efficacy. Results show that on day 21, rats treated with M. hirtus ointment had the highest percentage closure (94.5%) while honey treated and non-treated recorded 90.0% and 83.3% respectively. Similarly, a significant difference ($p < 0.05$) was observed on day 21 in the total collagen deposited in wounds of diabetic rats (10.57 ± 0.7) and *M. hirtus* ointment treated wounds (11.77 ± 0.4) as compared with the non-treated diabetic rats. M. hirtus ointment was efficacious in healing wounds in diabetic rats and heals wound faster than honey and may hold potential for wound healing in diabetes mellitus sufferers. However, the wound healing mechanism of this ointment needs further investigation.

Keyword: Collagen, diabetic rats, honey, *Mitracarpus hirtus,* ointment, wound healing

Abbreviations: DM: Diabetes Mellitus; STZ: Streptozotocin; DMSO: dimethyl sulphuroxide; DC: Diabetic Control; DHT: Diabetic honey treatment; DOT: Diabetic ointment treatment; NSSC: Neutral salt-soluble collagen; PSC: Pepsin soluble collagen; ASC: Acid soluble collagen

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in secretion of insulin, insulin action, or both (ADA 2013). There are three broad categories: type 1, type 2, and gestational diabetes. Although there are other types that are specific to other individual causes (Masharani and German 2011). Symptoms of DM include hyperglycemia, polyuria, polydipsia, and weight loss; sometimes with polyphagia, blurred vision autonomic and peripheral neuropathy which causes poor healing wounds (IDF 2011; Yamany and Sayed 2012).

A wound is defined as a breakdown in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue following injury to the skin or underlying tissues/organs caused by surgery, a blow, a cut, chemicals, heat/cold, friction shear force, pressure or as a result of disease (Shankar et al. 2014). In the treatment of chronic and other wounds, the primary goals are rapid wound closure and a functional and aesthetically satisfactory scar (Shankar et al. 2014). The wound proceeds through a normal healing cascade of four programmed phases, viz: Homeostasis, inflammation, proliferation and remodelling with specific events to achieve scar formation.

According to Guo and DiPietro (2010), these wound healing events involve: (i) rapid hemostasis; (ii) appropriate inflammation; (iii) mesenchymal cell differentiation, proliferation, and migration to the wound site; (iv) suitable angiogenesis; (v) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and (vi) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue.

Although the phases and events in wound healing are programmed, healing of wounds may delay. Microbial infections, exercise patterns, stress, nutritional status, and diseases such as diabetes have been identified as causes of delay (Damie 2011). Inflammatory and proliferative phases are the most affected stages during delay (Kurahashi and Fujii 2015). These two phases are characterized by neutrophils, macrophages, cytokines, keratinocytes, and fibroblasts, which ultimately result in collagen deposition and transformation of the extracellular matrix (Wong et al. 2013; Olczyk et al. 2014). Assessing the activities of these parameters is informative on progress of wound healing at a molecular level (Zaja-Milatovic and Richmond 2008; Kim et al. 2011; Upadhyay et al. 2014), but assessment of the end product (collagen) is more valuable, in that it is informative on the progress of healing and confirms the expected end products availability (Olczyk et al. 2014). This study used percentage wound closure, collagen deposition, and solubility in various solvents to compare the efficacy of a new herbal formulation (*M. hirtus* ointment) against honey in diabetic rats.

MATERIALS AND METHODS

Preparation of plant extract and ointment

The plant ointment was prepared according to the method detailed in Jato et al. (2018b). Briefly, harvested and identified *Mitracarpus hirtus* (L.) DC. (syn. *Mitracarpus villosus* (Sw.) Cham. & Schltdl. ex DC.) plants were washed and air-dried for 15days. Dried leaves were ground with a Linsan® blending machine (Lin 319) at 1000 RPM for 10 minutes at 25 ^{0}C and methanolic extraction achieved at 250 atm in a rotary evaporator (IKA® EW-28710-22). (50% w/w) *M. hirtus* ointment was prepared by heating 8 g of soft white paraffin in hot water bath for 30 min to melt. Then 8 g of the *M. hirtus* methanolic leave extract was dissolved in 20 mL dimethyl sulphur oxide (DMSO) from JHD® Ltd and mixed thoroughly with the melted paraffin for 20 min. 500 g of the dried leave were ground into fine powder with a Linsan® blending machine (Lin 319) at 1000 RPM for 10 minutes at 25°C.

Source and nature of honey used

500 mL of honey was purchased from a local honey farmer from Kwande Local Government area in Benue State and used for the entire research in its raw undiluted state, although no physicochemical analysis was done on the honey, the honey had a dark brown color and was very thick and sticky especially when allowed to make contact with air for a while.

Experimental animals

Care of animals was in line with National Institute of Health, guide for care and use of laboratory animals (Institute for Laboratory Animal Research 1996). Albino rats purchased from Benue State University Animal House weighing 160-250 g were allowed to acclimatize for 7 days prior to the initiation of the experiment in plastic cages and under laboratory conditions (temperature 22 ± 2 ^{0}C and 12 hr light-dark cycle). Animals were fed with balanced diet purchased from UAC foods Nigeria Ltd and water *ad libitum*.

Experimental design

In this Randomized controlled trial (RCT) study design (3^3), the rate of wound healing based on reduction in wound size and collagen quantification was studied for 21 days with 27 rats on days 7, 14 and 21. The rats were grouped into 3 (n=3) where group 1= control diabetic rats (non treated), group 2= diabetic rats (Honey treated), group =3 diabetic rats ointment treated.

Induction of diabetes mellitus and wound infliction

In fig 1 the flow chart for diabetes induction and wound healing study is presented. According to the method employed by Jato et al. (2018a), 12-hr fasted rats were administered single 65 mg/kg intraperitoneal injection of Streptozotocin (STZ) freshly prepared in 0.1 M sodium citrate buffer (pH 4.5). On day 8 after STZ injection, blood glucose measurement was performed on tail-vein blood with a glucometer (Accu-Chek® Aviva). Rats whose glucose tolerance test and fasting blood glucose levels exceeded 250 mg/dL (13.9 mmol/dL) were considered diabetic, consistent with the findings of (Mendes et al. 2012). The diabetic rats were then inflicted wounds same day to study wound healing by inflicting single full-thickness wounds (2 cm^2) on the dorsum of the rats after disinfection of area with ethanol 90% (Wong et al. 2011).

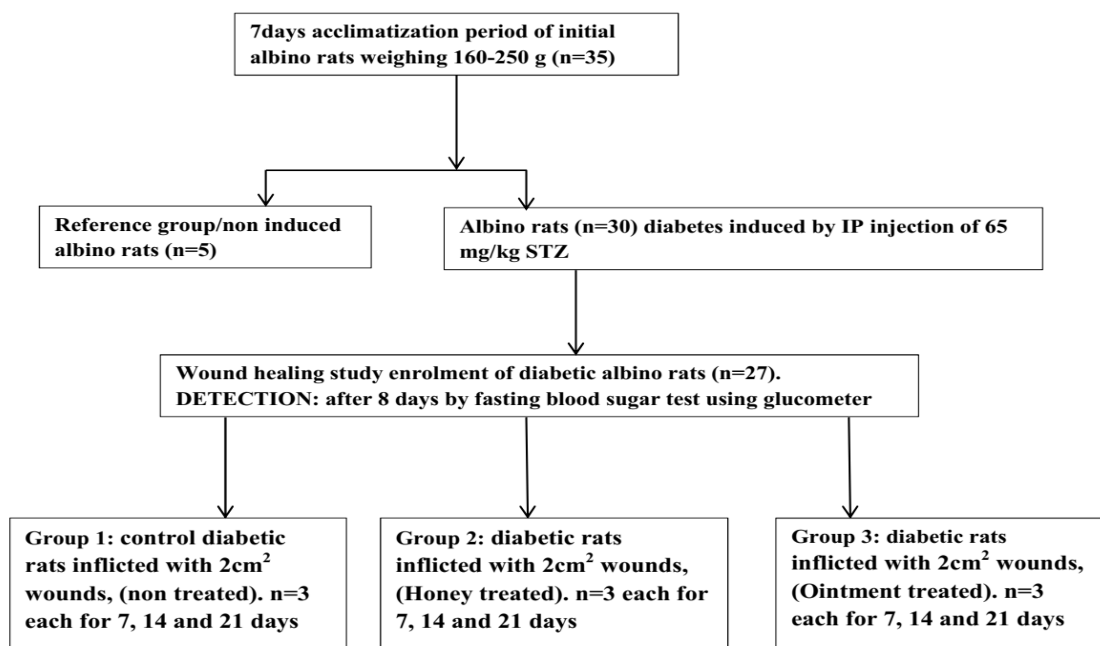

Figure 1. Flow chart for diabetes induction and wound healing study

Physical examination by measurement of wound size

Wounds were observed postoperatively on days 7, 14 and 21 by measurement of wound size with a rule to determine wound area and then calculating percentage closure as in (eq. 1). Granulated tissues were collected same day for collagen quantification as a measure of wound healing in the diabetic rats as described in Desallais et al. (2014).

$$\% \, wound \; closure = \frac{IWA - CWA}{IWA} \times 100 \quad \text{.......... (Eq.1)}$$

Where: IWA: initial wound area; CWA: current wound area

Collagen assay of granulation tissue

The total collagen content in wounds and solubility of collagen in different solvents obtained from granulation tissues in wounds was evaluated by the hydroxyproline assay method of Bergman and Loxly employed by Desallais et al. (2014).

Estimation of total collagen content in granulation tissue

The collagen content in wounds was evaluated by the hydroxyproline assay method of Bergman and Loxly (Desallais et al. 2014). After digestion of punch biopsy specimens (3 mm diameter) in 6 M HCl for 3 hr at 120°C, evaporated to dryness and then made up with a known volume of water. The pH of the samples was adjusted to 7.0. Afterward, samples were mixed with 0.06 M chloramine T and incubated for 20 min at room temperature. Then, 3.15 M perchloric acid and 20% dimethylaminobenzaldehyde added, and samples were incubated for an additional 20 min at 60°C. The absorbance was determined at 557 nm with a spectrophotometer (UV-vis Jenway® 7305).

Extraction of neutral salt-soluble collagen

Granulation tissue was minced, homogenized in 10 vol of neutral salt solvent (1.0 M NaCl, 0.05 M Tris, pH 7.5) containing 20 mM EDTA and 2.0 mM N-ethyl maleimide and stirred for 24 hr. The suspension was then centrifuged at 35,000 g for 1 hr at 4 °C and the extraction was repeated with the pellet. The supernatants were pooled and an assay of hydroxyproline was done.

Extraction of acid-soluble collagen

The residue obtained from neutral salt soluble collagen was re-suspended in 10 vol of 0.5 M acetic acid and extracted for 24 hrs and centrifuged at 5000 g for 10 min. The pellet was then extracted with acetic acid, supernatants were pooled and an aliquot then used for the determination of hydroxyproline as in neutral salt soluble collagen.

Extraction of pepsin-soluble collagen

The residue obtained after acid extraction was re-suspended in 0.5 M acetic acid containing 100 mg pepsin per g of wet tissue. Digestion was carried out for 24 hr followed by centrifugation at 5000 g for 10 min and re-extraction. Aliquots of pooled supernatant were then used for hydroxyproline measurement and aldehyde content in the collagen.

Statistical analysis

SPSS software version 21 was used for analysis of results and is expressed as mean ± S.D. Means were compared statistically using ANOVA and Tukey test as post hoc test. A statistically significant p-value <0.05 was considered.

RESULTS AND DISCUSSIONS

Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by % wound closure

In Figure 2, wound healing results in albino rats treated as measured by percentage wound closure are presented. On day 7, the diabetic rats treated with *M. hirtus* ointment recorded the highest percentage closure of 14.50% while rats treated with honey recorded the least closure (3.3%). On day 14, the non-treated diabetic group (16.3%) whereby they showed lesser percentage of wound closure than the honey-treated rats (32.5%) while the *M. hirtus* ointment treated group maintained the lead with a percentage closure of 45.0%. On day 21, the diabetic rats treated with *M. hirtus* ointment maintained highest percentage closure of 94.5%. The change observed on day 14 was also maintained, an 83.3% and 90.0% wound closure was recorded for non-treated diabetic rats and diabetic rats treated with honey respectively. In all, wound closure and scar formation were recorded on days 16, 17 and 19 for diabetic ointment treatment, diabetic honey treatment, and diabetic control.

Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by assessment of collagen formation

Table 1 presents results of total collagen deposited in wounds of treated and non-treated diabetic rats. They show that there is no statistically significant ($p < 0.05$) increase in the total collagen deposited in wounds of diabetic rats treated with *M. hirtus* ointment and with honey at day 7 post wound excision. A statistically significant difference ($p < 0.05$) was observed at day 14 in the deposition of total collagen for diabetic rats treated with honey (5.53 ± 0.2) and diabetic rats treated with *M. hirtus* ointment (5.67 ± 0.3) as compared with the non-treated diabetic rats (5.00 ± 0.1). Similarly, a significant ($p < 0.05$) increase was observed at day 21 in the deposition of total collagen in honey-treated wounds of diabetic rats (10.57 ± 0.7) and *M. hirtus* ointment treated wounds (11.77 ± 0.4) as compared with the non-treated diabetic rats. Thus all diabetic rats had increased total collagen formation with increased days of healing.

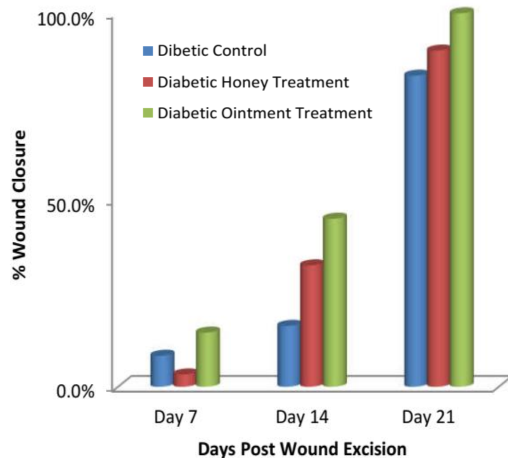

Figure 2. Wound healing in albino rats treated as measured by percentage wound closure

Table 1. Total collagen deposited at 7, 14, and 21 days post wound excision in STZ-induced diabetic rats in various treatment groups

Group	Duration of wounds		
	Day 7	Day 14	Day 21
DC	1.70 ± 0.4^{a}	5.00 ± 0.1^{a*}	9.83 ± 0.4^{a*}
DHT	1.93 ± 0.9^{a}	5.53 ± 0.2^{b}	10.57 ± 0.7^{ab}
DOT	2.40 ± 0.4^{a}	5.67 ± 0.3^{b}	11.77 ± 0.4^{b}

DC-Diabetic Control, DHT-Diabetic honey treatment, and DOT-Diabetic ointment treatment. Data are expressed as the mean ± SD from three animals in each group. Significant differences between the diabetic control and treated groups were assessed using Turkey test. *: significant difference ($p < 0.05$), $^{a, b}$: values with same alphabets belongs to the same Homogeneous subset

Tables 2, 3 and 4 show solubility of collagen in various solvents namely: acid, neutral salt and pepsin respectively for the treated and non-treated groups. Results show that, no significant ($p < 0.05$) decrease was recorded in the pepsin soluble collagen (PSC) and neutral salt soluble collagen (NSSC) at day 7, 14 and 21 in the diabetic rats treated with honey, and diabetic rats treated with *M. hirtus* ointment when compared to the non-treated diabetic rats. Conversely, a significant ($p < 0.05$) decrease was observed in the solubility of acid-soluble collagen (ASC) on days 7, 14 and 21 in the diabetic rats treated with honey, and diabetic rats treated with *M. hirtus* ointment when compared to the non-treated diabetic rats. There was a decreasing trend in the solubility of ASC, NSSC and PSC in all the groups, indicating maturation and toughness of collagen deposits through the healing period. Therefore, in all the groups of diabetic rats there was a decrease in solubility with increase in days of experiment.

The *M. hirtus* ointment-treated diabetic rat wounds showed higher percentage closure than the honey-treated diabetic wounds in the albino rats while the non-treated diabetic rat wounds recorded the least percentage closure. The findings agree with that of Dwivedi and Chaudhary (2012) on ampucure in diabetic wounds in rats while the findings of Barua et al. (2013) (89.76%) agree with findings using honey on day 21. Also, Sazegar et al. (2011) and Nisbet et al. (2010) reported a significant percentage closure in honey-treated wounds than the non-treated wounds.

Table 2. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in acid

Days	7	14	21
Group 1: DC (Mean ± SD)	524.00 ± 11.10^{b}	506.00 ± 9.00^{b}	492.67 ± 11.40^{b}
Group 2: DHT (Mean ± SD)	513.67 ± 5.70^{b*}	499.00 ± 7.00^{b}	473.67 ± 13.10^{b*}
Group 3: DOT (Mean ± SD)	393.67 ± 11.00^{a*}	356.67 ± 49.00^{a*}	342.00 ± 44.00^{a*}

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ($^{a, b}$) belong to the same Homogeneous subset as compared by Turkey post Hoc test

Table 3. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in neutral salt

Days	7	14	21
Group 1: DC (Mean ± SD)	116.67 ± 16.80^{a}	92.33 ± 10.80^{a}	78.67 ± 8.10^{a}
Group 2: DHT (Mean ± SD)	106.0 ± 4.00^{a}	85.33 ± 13.60^{a}	73.00 ± 9.60^{a}
Group 3: DOT (Mean ± SD)	101.33 ± 6.80^{a}	80.67 ± 13.60^{a}	68.33 ± 9.00^{a}

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ($^{a, b}$) belong to the same Homogeneous subset as compared by Turkey post Hoc test

Table 4. Wound healing in treated and non-treated STZ-induced diabetic albino rats as indicated by solubility of collagen in pepsin

Days	7	14	21
Group 1: DC (Mean ± SD)	2730.33 ± 333.00[a]	2480.00 ± 385.10[a]	2349.00 ± 201.80[a]
Group 2: DHT (Mean ± SD)	2483.00 ± 404.50[a]	2266.33 ± 185.00[a]	2188.33 ± 424.90[a]
Group 3: DOT (Mean ± SD)	2278.67 ± 294.40[a]	2075.33 ± 168.20[a]	1996.00 ± 314.00[a]

Note: DC-Diabetic Control, DHT-Diabetic honey treatment, DOT-Diabetic ointment treatment. *: significantly different down the column at $p < 0.05$ when compared to the control, Values with the same alphabetical superscripts ([a, b]) belong to the same Homogeneous subset as compared by Turkey post Hoc test

The higher percentage closure observed in the treated wounds of the diabetic rats can be attributed to increased deposition of total collagen and reduction in oxidative stress arising from the infliction of wounds to the rats. Close interaction of cells is necessary for synthesis and deposition of collagen facilitated by contracture of the skin tissue (Lembong et al. 2017). Cardiac glycosides have been implicated for contraction in tissues by inhibiting the Na^+-K^+ ATPase pump in the sarcolemmal membrane of the myocyte and other cells (Cunningham et al. 2018). This inhibition causes intracellular accumulation of Na^+, which makes the Na^+-Ca^{2+} pump extrude less Ca^{2+}, causing Ca^{2+} to accumulate inside the cell resulting in increased force of contraction necessary for wound closure (Lembong et al. 2017). In addition to excellent water-absorbing quality, biodegradability, biocompatibility and outstanding film-forming properties, interactions of Ca^{2+} ions with the cells to facilitate contractions are the basis for using calcium alginate in wound dressing (Wang et al. 2015). Therefore, we submit that increased closures associated with the plant ointment may be the effect of cardiac glycosides in the plant on the wounds.

Collagen obtained from the treated and non-treated diabetic rats showed decreased solubility in the various solvents through the days of treatment. Also, the collagen of *M. hirtus* and honey exhibited lesser solubility in the acid, neutral salt buffer and pepsin solvents. This decrease in solubility was a result of collagen maturation (Weston and Althen 2002). The mature collagens have lesser crosslinks because the initial collagen produced is reducible and over time are replaced by mature, thermally stable and less soluble crosslinks. These mature crosslinks rather than total collagen are the key factors in collagen-related toughness (Weston and Althen 2002; Ponrasu and Suguna 2014). In an earlier submission, we reported that there was decreased antioxidant enzyme activity associated with increased wound healing. The development of mature tough collagen and the epidermal layer stratum corneum have been implicated for this negative correlation and the resultant challenges in transdermal drug delivery (Yang et al. 2018; Jato et al. 2021). Results obtained for the acid-soluble collagen were in agreement with the findings of Ponrasu and Suguna (2014) and Ponrasu et al. (2018) while that of the NSSC was in agreement with reports by Kirubanandan et al. (2013) and Ponrasu and Suguna (2014). PSC also recorded very high solubility more than the ASC and NSSC as observed by other studies (Ponrasu and Suguna 2014; Ponrasu et al. 2018; Kirubanandan et al. 2013). This is because the acid and the neutral salt only carry out partial solubility, while the insoluble collagen is soluble in pepsin (Kirubanandan et al. 2013).

In conclusion, from the findings in this study, it was concluded that wounds of diabetic rats treated with *M. hirtus* ointment healed faster than honey. Thus diabetic rats treated with the ointment recorded the highest percentage of wound closure. Also, total collagen deposited in diabetic rats was highest in the ointment treated rats followed by honey treated rats while the non-treated was least and the solubility of collagen in the various solvents was decreased concomitantly with increase in healing and was lesser in the ointment treated diabetic rats, suggesting faster maturation of the deposited collagen. It is our view however that, the mechanism of action of the ointment be studied in addition to the identification and isolation of the bioactive compounds responsible for the efficacy of the ointment.

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Anticancer mechanism of artonin E and related prenylated flavonoids from the medicinal plant *Artocarpus elasticus*

CHRISTIAN BAILLY
OncoWitan, Scientific Consulting Office. Lille (Wasquehal), 59290, France. ♥email: christian.bailly@oncowitan.com

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Abstract. *Bailly C. 2021. Anticancer mechanism of artonin E and related prenylated flavonoids from the medicinal plant* Artocarpus elasticus. *Asian J Nat Prod Biochem 19: 44-56.* Plants of the *Artocarpus* genus are largely distributed throughout tropical Asia and Oceania. Species such as *A. altilis* (Parkinson) Fosberg and *A. heterophyllus* Lam. are popular trees known as breadfruit and jackfruit, respectively. They contain a large structural diversity of bioactive prenylated flavonoids. Here we have focused on the less known species *Artocarpus elasticus* Reinw. ex Blume which is well distributed in southeast Asia, and used in traditional medicine to treat dysentery, tuberculosis, and other diseases. Numerous prenylated flavonoids have been isolated from the leaves and bark of *A. elasticus*, such as artocarpesin, artocarpin, artelastin, and many others. They are endowed with antioxidant, anti-inflammatory, and anticancer properties. A focus is made of the subgroup of compounds designated artonins, with the derivative artonin E as a lead anticancer agent. Art-E has revealed marked anticancer effects *in vitro* and *in vivo*, after oral administration. The mechanism of action of Art-E is discussed, to highlight the structural and functional analogy between Art-E and the antitumor natural product morusin. Both compounds trigger TRAIL-mediated apoptosis of cancer cells. They can be considered further for the development of novel anticancer agents.

Keywords: *Artocarpus*, cancer therapeutics, natural products, phytotherapy, prenylated flavonoids

Abbreviations: iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species

INTRODUCTION

The genus *Artocarpus* refers to a large group of plants belonging to the Moraceae family (Zerega et al. 2010). The internet database www.theplantlist.org has recorded 68 taxonomically accepted species, while about 90 additional plant names are now in synonymy or have even been moved as synonyms to other genera. The name *Artocarpus* derives from the Greek *artos* for "bread" and *karpos* for "fruit". Some of the *Artocarpus* species are relatively well-known, such as *A. altilis* (Parkinson) Fosberg known as the breadfruit tree, and *A. heterophyllus* Lam. known as the jackfruit tree, which are two economically important *Artocarpus* species (Sikarwar et al. 2014; Mohammed Haleel et al. 2018; Sahu et al. 2019; Buddhisuharto et al. 2021). Their fruits are used for food production and several parts of these two trees including fruits, leaves, and barks have been used in traditional medicine in India and surrounding countries (Ranasinghe et al. 2019). Other *Artocarpus* species are used in traditional medicine, such as (i) decoctions prepared from the leaves of the tree *A. tonkinensis*, used in northern Vietnam to treat arthritis and backache (Adorisio et al. 2016), and (ii) extracts of *Artocarpus lacucha* (also known as Monkey jack) used in traditional Thai medicine as an anthelmintic agent (Aneklaphakij et al. 2020; Gupta et al. 2020), for examples. The ethnopharmacological use of *Artocarpus* species is widespread in subtropical and tropical regions of Asia where the trees are largely distributed. *Artocarpus* are believed to originate from the island of Borneo, from which species dispersed and diversified in several directions (Williams et al. 2017). *Artocarpus*-based remedies are used to treat multiple diseases and conditions, including malarial fever, diarrhea, diabetes, parasitic infections, and different inflammatory diseases (Jagtap and Bapat 2010).

These plants contain a large diversity of bioactive compounds, including many prenylated flavonoids and cyclized derivatives which have revealed interesting antimicrobial, anti-inflammatory and anticancer properties (Hakim et al. 2006; Hari et al. 2014). One particular species, *Artocarpus elasticus* Reinw. ex Blume (Figure 1) has shown marked anticancer effects against different cancer cell lines and tumor models, attributed to the presence of prenylated flavonoids isolated from the root bark or other parts of the tree. The lead compound is artonin E (Art-E), thoroughly investigated as an antitumor agent. Over the past ten years, the mechanism of action of Art-E has been delineated and the activated signaling pathways at the origin of antitumor activity have been defined. These studies gave us the impetus to analyze in deep the mode of action of Art-E and structurally related compounds. The present review will address successively the plant *A. elasticus* and plant extracts, the phytochemical compounds isolated from the plant, the specific flavonoid group known as artonins, and the molecular targets and pathways activated by Art-E, responsible for its pharmacological properties. The role of TRAIL in the mode of action of Art-E and related compounds is discussed, opening the door to the identification of novel anticancer molecules. The purpose of the study was to promote the knowledge of artonins and the use of artonin E as a lead anti-cancer compound.

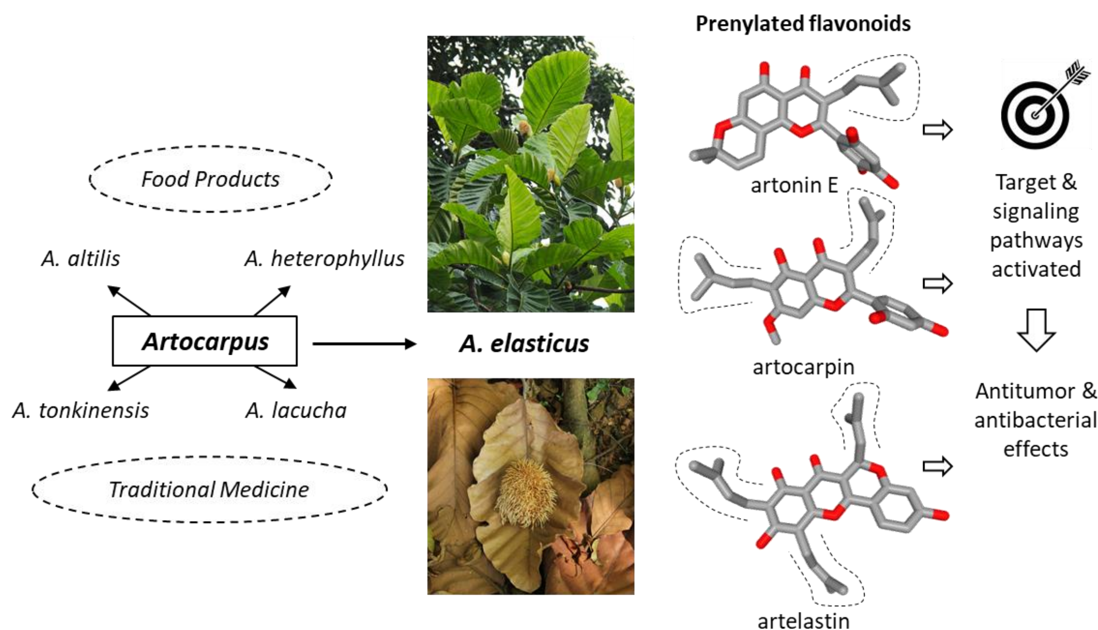

Figure 1. *Artocarpus elasticus* Reinw. ex Blume as a source of anticancer prenylated flavonoids. Different *Artocarpus* species are used in traditional medicine and as food products, such as *A. altilis* (Parkinson ex F.A.Zorn) Fosberg, *A. heterophylus* Lam., *A. tonkinensis* A.Chev. ex Gagnep. and *A. lacucha* Buch.-Ham. Similarly, *A. elasticus* is also a source of bioactive mono-, bis- and tris-prenylated flavonoids, endowed with anticancer properties. *A. blumei* Trécul and *A. kunstleri* King are synonyms for *A. elasticus* Reinw. ex Blume (http://www.theplantlist.org/)

ARTOCARPUS ELASTICUS AND ITS MEDICINAL USES

The plant *Artocarpus elasticus* Reinw. ex Blume is native to Southeast Asia and can easily be found in countries like Myanmar, Thailand, Malaysia, Indonesia and the Philippines. In some countries like Malaysia and Indonesia, *A. elasticus* is cultivated to maintain its sustainability (Susiarti et al. 2020). It is an evergreen, robust and tall tree (up to 45-65 m). Various vernacular names are used locally, such as Benda (Javanese Indonesia), Terap nasi (Peninsular Malaysia), and others (Teo and Nasution 2003)

The wood of *A. elasticus is sought* as a building material. Upon wounding, it exudes a white and thick latex which is elastic or flexible, hence the name *elasticus*. The sticky latex is used to catch birds but also to treat dysentery. Different parts of the tree are used in traditional medicines, the latex for dysentery, the bark for female contraception, and the young leaves mixed with rice to treat tuberculosis. The flesh of the fruits is eaten raw or cooked. The seeds are consumed fried or roasted (Susiarti et al. 2020).

The bark and the leaves of the plant are often used to identify new phytochemicals. In addition, an extract of the stem bark of *A. elasticus* has been used as a reducing and stabilizing agent for the synthesis of silver nanoparticles (Abdullah et al. 2015). The same type of antibacterial silver nanoparticles have been obtained with seed extract of *A. hirsutus* (Shobana et al. 2020).

PRENYL FLAVONOIDS FROM *ARTOCARPUS ELASTICUS*

Artocarpus elasticus is a rich reservoir of bioactive natural products, notably prenyl flavonoids particularly abundant in the bark of the tree and the roots. There is a large structural diversity of prenylated flavonoids isolated from *A. elasticus,* with compounds bearing one, two or three prenyl groups, with a classical flavonoid skeleton or a bulkier pentacyclic core (Nomura et al. 1998; Kijjoa et al. 1998; Cidade et al. 2001). The main isolated compounds are shown in Figure 2, with the exception of the artonins discussed below. Most of these compounds can be found in other *Artocarpus* species. Here, we will discuss only those found in *A. elasticus.*

The tetracyclic compound artelastin, with three prenyl side chains, has been isolated in 1996 from the wood of *A. elasticus together with* artelastochromene, artelasticin, and artocarpesin (Kijjoa et al. 1996). Artelastin has revealed marked cytotoxic properties against different cancer cell lines *in vitro*. It was found to disturb the microtubule network of MCF7 breast cancer cells and to interfere with the cell cycle progression. Artelastin caused an accumulation in S phase due to drug-induced delay in DNA replication (Pedro et al. 2005). It is a potent inhibitor of both T- and B lymphocyte mitogen-induced proliferation and an inhibitor of cytokines production, such as interferon-γ, interleukins IL-2, -4 and -10, in stimulated splenocytes (Cerqueira et al. 2003). Artelastin displays marked antioxidant properties, inhibiting the production of reactive oxygen species (ROS) and the expression of the inducible nitric oxide synthase (iNOS) in lipopolysaccharide-stimulated macrophages (Cerqueira et al. 2008).

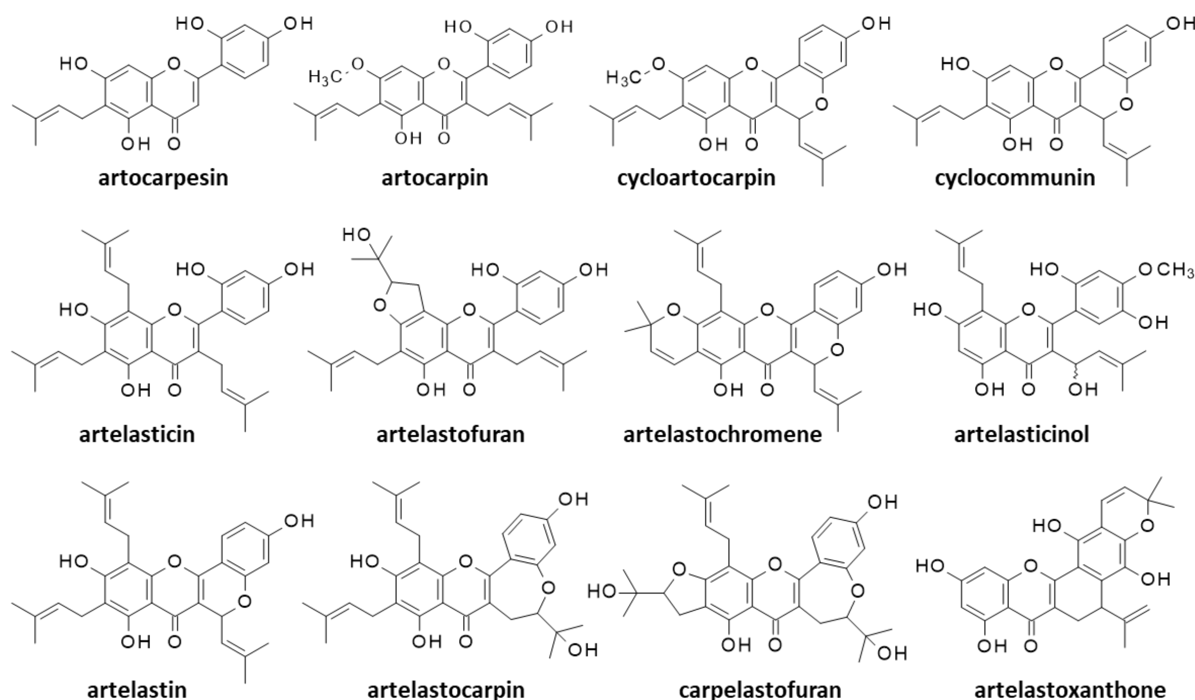

Figure 2. Chemical structures of prenylated flavonoids which have been isolated from *A. elasticus* Reinw. ex Blume

Artelasticin can be found in *A. elasticus* (Kijjoa et al. 1996), in *A. lanceifolius* (Syah et al. 2001) *and other plants, such as the* Cameroon medicinal plant *Dorstenia psilurus*. It was found to activate AMPK (AMP-activated protein kinase) and stimulate glucose uptake in rat skeletal muscles. Its marked blood glucose-lowering effect can be useful for the treatment of type-2 diabetes (Choumessi et al. 2019). Artocarpesin was found to be moderately cytotoxic toward a panel of cancer cell lines (IC_{50} values in the 60-100 μM range) but its analog cycloartocarpesin is more potent (IC_{50} values in the 15-50 μM range) (Kuete et al. 2015). This compound can be found in many other plants. Like artelasticin, it presents marked anti-inflammatory and antioxidant properties (Fang et al. 2008). Its antioxidant activity contributes to the anti-browning effect observed with extracts of *A. heterophyllus* on fresh-cut apple slices. Remarkably, artocarpesin was found to exert a powerful inhibitory activity against mushroom tyrosinase (IC_{50} = 0.52 μM) whereas the related product artocarpin was totally inactive against the enzyme (Zheng et al. 2008, 2009). Artocarpesin, found in the twigs and woods of *A. heterophyllus*, *A. incisus,* and *A. elasticus*, has been considered for the design of skin whitening agents (Arung et al. 2008, 2011). Artocarpin has been reported in a dozen of *Artocarpus* species and displays antioxidant, anti-inflammatory and anti-parasitic properties, as well as skin-whitening activities (Chan et al. 2018a; Morrison et al. 2021).

The bis-prenyl flavonoid cyclocommunin is known for a long time for its potent capacity to inhibit platelet aggregation induced by collagen or arachidonic acid (Lin et al. 1993). It was first isolated from *A. communis*, hence its name cyclocommunin (Lin and Shieh, 1992), but it is also called isocyclomulberrin (Chen et al. 1993; Ma et al. 2010). Cyclocommunin is also an activator of protein kinase C (PKC) and a compound capable to elevate the level of intracellular calcium in rat neutrophils. As a result, cyclocommunin was found to stimulate respiratory burst in neutrophils (Wang et al. 1999), enhancing the production of superoxide anion, unlike artonin B which inhibited superoxide anion formation in these cells (Wei et al. 2005). Cyclocommunin displays modest anti-proliferative effects against hepatocellular carcinoma cells (IC_{50} = 14-33 μM depending on cell line) whereas Art-A and Art-B were inactive against these HCC cell lines (Ma et al. 2010). Recently, it was reported that cyclocommunin displays a significant antimycobacterial activity against *Mycobacterium tuberculosis* (laboratory strain H37Ra) with a minimum inhibitory concentration (MIC) of 12.3 μM (Boonyaketgoson et al. 2020).

Artelastocarpin and carpelastofuran were isolated from the wood of *A. elasticus*. They both revealed cytotoxic properties toward cancer cells, with an efficacy like that of artelastin, artelasticin, and artlastochromene (IC_{50} = 7-12 μM depending on cell lines) (Cidade et al. 2001). A series of compounds named artoindonesianins has been obtained from different *Artocarpus* species (Musthapa et al. 2010). Most of them have been isolated from species other than *A. elasticus*, such as the cytotoxic compounds artoindonesianins Z-4 and Z-5 found in the bark of *A. lanceifolius* (Musthapa et al. 2009a) and artoindonesianins A and B found in the roots of *A. champeden* (Hakim et al. 1999) and many other artoindonesianins, as indicated in Table 1.

Table 1. Artoindonesianin compounds isolated from *Artocarpus* species

Compound	*Artocarpus* species	References
Artoindonesianin A	*A. champeden*	Hakim et al. 1999
Artoindonesianin B	*A. champeden*	Hakim et al. 1999
Artoindonesianin C	*A. teysmanii*	Makmur et al. 2000
	A. rigidus	Namdaung et al. 2006 ;
	A. kemando	Ee et al. 2011
Artoindonesianin D	*A. maingayi*	Hakim et al. 2000
Artoindonesianin E	*A. champeden*	Hakim et al. 2001
Artoindonesianin F	*A. heterophyllus*	Rao et al. 2010
Artoindonesianin G	*A. lanceifolius*	Syah et al. 2001
Artoindonesianin H	*A. lanceifolius*	Syah et al. 2001
Artoindonesianin I	*A. lanceifolius*	Syah et al. 2001
Artoindonesianin J	*A. bracteata*	Ersam et al. 2002
Artoindonesianin K	*A. champeden*	Achmad et al. 2005
Artoindonesianin L	*A. rotunda*	Suhartati et al. 2001
Artoindonesianin M	*A. champeden*	Syah et al. 2002b
Artoindonesianin N	*A. gomezianus*	Hakim et al. 2002a
Artoindonesianin O	*A. gomezianus*	Hakim et al. 2002a
Artoindonesianin P	*A. lanceifolius*	Hakim et al. 2002b
	A. elasticus	Jenis et al. 2019
Artoindonesianin Q	*A. champeden*	Syah et al. 2002a
Artoindonesianin R	*A. champeden*	Syah et al. 2002a
Artoindonesianin S	*A. champeden*	Syah et al. 2002a
Artoindonesianin T	*A. champeden*	Syah et al. 2002a
Artoindonesianin U	*A. champeden*	Syah et al. 2004
Artoindonesianin V	*A.. champeden*	Syah et al. 2004
	A. altilis	Shamaun et al. 2010
Artoindonesianin W	*A. elasticus*	Jenis et al. 2019
Artoindonesianin X	*A. fretessi*	Soekamto et al. 2003
Artoindonesianin Y	*A. fretessi*	Soekamto et al. 2003
Artoindonesianin Z1	*A. lanceifolius*	Hakim et al. 2006
	A. anisophyllus	Noraini et al. 2013
Artoindonesianin Z2	*A. lanceifolius*	Hakim et al. 2006
Artoindonesianin Z3	*A. lanceifolius*	Hakim et al. 2006
Artoindonesianin Z4	*A. lanceifolius*	Musthapa et al. 2009b
Artoindonesianin Z5	*A. lanceifolius*	Musthapa et al. 2009b
Artoindonesianin Z4	*A. lanceifolius*	Musthapa et al. 2009b
Artoindonesianin Z5	*A. lanceifolius*	Musthapa et al. 2009b
Artoindonesianin A1	*A. champeden*	Syah et al. 2006b
Artoindonesianin A2	*A. champeden*	Syah et al. 2006a
Artoindonesianin A3	*A. champeden*	Syah et al. 2006a
Artoindonesianin B1	*A. altilis*	Syah et al. 2006b
	A. heterophyllus	Lang et al. 2016
Artoindonesianin E1	*A. elasticus*	Musthapa et al. 2009a

For the record, the structure of artoindonesianin C, first isolated from *A. teysmanii* (Makmur et al. 2000), has been printed on a post stamp on the occasion of the international year of chemistry 2011 (Figure 3). The oxepinoflavone derivative artoindonesianin E1 was isolated from the bark of *A. elasticus*, together with the related compounds artocarpin, cycloartocarpin, and cudraflavones A and C (Figure 2). The derivative artoindonesianin A-3 was initially isolated from *A. champeden* (Syah et al. 2006a) and latter re-discovered from the bark of *A. elasticus* tree collected from Alor Island in Indonesia (Kuran and Ersam 2017). Other artoindonesianins have been found in *A. elasticus*, such as artoindonesianin P which was found to inhibit the enzyme α-glucosidase (IC_{50} = 25.4 μM) but with a reduced efficacy compared to Art-E and artobiloxanthone (IC_{50} = 16.2 and 8.6 μM, respectively) (Jenis et al. 2019). Recently, artoindonesianins P and W were isolated from *A. elasticus* and found to inhibit human neutrophil elastase (IC_{50} = 28.7 and 11.2 μM, respectively) whereas artobiloxanthone was slightly more active (IC_{50} = 9.8 μM) (Ban et al. 2020).

Other prenylated flavonoids have been identified, such as artelastoheterol, artelasticinol, artelastoxanthone, artobiloxanthone, cycloartelastoxanthone, cycloartobiloxanthone, cycloartelastoxanthendiol, elastixanthone, and artonol A, all isolated from the root bark of *A. elasticus* (Figure 2). Artelastoxanthone has revealed modest antiproliferative activities against different cancer cell lines *in vitro* (Ko et al. 2005). Cycloartelastoxanthone and cycloartobiloxanthone and artobiloxanthone have shown protective effects on DNA damage caused by superoxide anion radicals $O_2^{\bullet-}$ (Lin et al. 2009). Elastixanthone and cycloartobiloxanthone have shown antimicrobial activities (Ramli et al. 2016). Artobiloxanthone and cycloartobiloxanthone have been found in many *Artocarpus* species, not limited to *A. elasticus*. The latter compound is interesting because it was found to potently inhibit migration and invasion of H460 lung cancer cells, via inhibition of the phosphorylation of focal adhesion kinase (FAK) and the expression of cell division cycle 42 (CDC42) (Tungsukruthai et al. 2017). Cycloartobiloxanthone dose-dependently reduces proliferation of other lung cancer cell lines (H23, H292 and A549) and triggers caspase-dependent apoptosis with an efficacy comparable to that of the standard DNA-damaging anticancer drugs cisplatin and etoposide, at least *in vitro* (Losuwannarak et al. 2018). But the ROS scavenging activity of cycloartobiloxanthone is much weaker than that of artobiloxanthone and Art-E (Sritularak et al. 2010). A recent molecular docking analysis has predicted that artobiloxanthone can bind to the active site of the enzyme transglutaminase 2 (TG2), possibly inhibiting this enzyme considered as an anticancer target (Parvatikar and Madagi 2021).

Figure 3. The structure of artoindonesianin C, isolated from *A. teysmanii*. A post stamp with the structure of the compound was emitted to celebrate the international year of chemistry 2011 in Indonesia

Here we focused mostly on prenylated flavonoids isolated from *A. elasticus* but the whole *Artocarpus* genus the structural diversity is huge, as previously reported in specific review articles on *Artocarpus* species (Achmad et al. 2005; Hakim et al. 2006; Hakim 2010; Veitch and Grayer 2008). A recent analysis of a root extract of *A. heterophyllus* led to the identification of 47 prenylated flavonoids (Ye et al. 2019). There must be that many in the related species *A. elasticus*. An important group of prenylated flavonoids commonly found in *Artocarpus* species is called artonin, with the lead compound Art-E. Given the importance of the lead, this family is presented hereafter.

THE ARTONIN GROUP OF PRENYL FLAVONOIDS

There are 24 compounds named artonins A to Y (Table 2). The first two compounds, Art-A and -B, were isolated in 1989 for the root bark of *A. heterophylus* (Hano et al. 1989) followed by Art-C and -D one year later from the same plant (Hano et al. 1990a). They can be found in a few other *Artocarpus* species and display antioxidant properties (Ko et al. 2005). Art-B is an interesting compound, much more potent than Art-A at inhibiting the proliferation of nasopharyngeal cancer KB cells and the growth of the parasite *Trypanosoma brucei brucei*, *in vitro* (Bourjot et al. 2010). Art-B can reduce proliferation of different types of cancer cells, such as human CCRF-CEM leukemia cells, via the induction of mitochondria-dependent apoptosis (Lee et al. 2006). Art-A is less cytotoxic than Art-B, nevertheless, it is a more potent inhibitor of the papain-like cysteine protease cathepsin K (IC_{50} = 1.9 and 9.0 μM, for Art-A and Art-B respectively) largely implicated in bone resorption (Zhai et al. 2017). Art-A also displays antimalarial activity, at least *in vitro* (Widyawaruyanti et al. 2007).

Table 2. Artonin compounds isolated from *Artocarpus* species

Compound	CID[a]	Formula (g/mol)[b]	*Artocarpus* species	References
Artonin A	14557102	$C_{30}H_{30}O_7$ (502.6)	*A. heterophylus*	Hano et al. 1989
			A. styracifolius	Bourjot et al. 2010
			A. hypargyreus	Qiao et al. 2011
			A. xanthocarpus	Jin et al. 2015
			A. champeden	Widyawaruyanti et al. 2007
Artonin B	11964501	$C_{30}H_{30}O_7$ (502.6)	*A. heterophylus*	Hano et al. 1989
			A. champeden	Hakim et al. 1999s
			A. styracifolius	Bourjot et al. 2010
Artonin C	14681571	$C_{40}H_{38}O_{10}$ (678.7)	*A. heterophylus*	Hano et al. 1990a
Artonin D	14681573	$C_{40}H_{36}O_{10}$ (676.7)	*A. heterophylus*	Hano et al. 1990a
Artonin E	5481962	$C_{25}H_{24}O_7$ (436.5)	*A. communis*	Hano et al. 1990b
			A. kemando	Seo et al. 2003
			A. lanceifolius	Cao et al. 2003
			A. chama	Wang et al. 2004
			A. nobilis	Jayasinghe et al. 2008
			A. lanceifolius	Musthapa et al. 2009
			A. gomezianus	Plaibua et al. 2013
			A. rotunda	Suhartati et al. 2001
			A. rigida	Suhartati et al. 2018
Artonin F	14680593	$C_{30}H_{30}O_7$ (502.6)	*A. rigidus*	Namdaung et al. 2006
			A. styracifolius	Bourjot et al. 2010
			A. integer var. silvestris Corner	Shaha et al. 2016
Artonin G	46887714	$C_{30}H_{30}O_7$ (502.6)	*A. rigida*	Hano et al. 1990
Artonin H	21595104	$C_{30}H_{34}O_7$ (506.6)	*A. rigida*	Hano et al. 1990
Artonin I	57335177	$C_{40}H_{36}O_{11}$ (692.7)	*A. heterophyllus*	Hano et al. 1992
Artonin J	44258663	$C_{25}H_{24}O_7$ (436.5)	*A. heterophyllus*	Aida et al. 1993
Artonin K	15340661	$C_{21}H_{18}O_7$ (382.4)	*A. heterophyllus*	Aida et al. 1993
Artonin L	44258662	$C_{22}H_{20}O_7$ (396.4)	*A. heterophyllus*	Aida et al. 1993
Artonin M	44258661	$C_{30}H_{30}O_7$ (502.6)	*A. altilis*	Hano et al. 1993
			A. rotunda	Suhartati et al. 2001
Artonin N	44258669	$C_{30}H_{30}O_7$ (502.6)	*A. rigida*	Hano et al. 1993
Artonin O	46887814	$C_{30}H_{30}O_7$ (502.6)	*A. rigida*	Hano et al. 1993
			A. rotunda	Suhartati et al. 2001
Artonin P	44258658	$C_{25}H_{20}O_8$ (448.4)	*A. rigida*	Hano et al. 1993
			A. communis	Chan et al. 2018b
Artonin Q	131753034	$C_{31}H_{30}O_8$ (530.6)	*A. heterophyllus*	Aida et al. 1994
Artonin R	131753035	$C_{31}H_{30}O_{10}$ (562.6)	*A. heterophyllus*	Aida et al. 1994
Artonin S	44258666	$C_{26}H_{28}O_7$ (452.5)	*A. heterophyllus*	Aida et al. 1994
Artonin T	44258664	$C_{26}H_{26}O_7$ (450.5)	*A. heterophyllus*	Aida et al. 1994
Artonin U	44258358	$C_{21}H_{20}O_5$ (352.4)	*A. heterophyllus*	Aida et al. 1994
Artonin V	129687399	$C_{25}H_{26}O_7$ (438.5)	*A. altilis*	Hano et al. 1994
Artonin X	(FDB021143)	$C_{40}H_{38}O_9$ (662.7)	*A. heterophyllus*	Shinomiya et al. 1995
Artonin Y	15541482	$C_{20}H_{18}O_6$ (354.4)	*A. heterophyllus*	Shinomiya et al. 2000

Note: [a]Compound Identity number (PubChem CID). [b]Formula and molecular weight.

The most important compound in the series is arguably Art-E (also known as 5'-hydroxymorusin), initially isolated from the stem bark of *A. communis* Forst., together with Art-F (Hano et al. 1990b). It was later found in several other *Artocarpus* species, including *A. elasticus* (Ramli et al. 2016). The compound, bearing an isoprenoid group at position *C-3*, was initially characterized as a potent and selective inhibitor of arachidonate 5-lipoxygenase (IC_{50} = 0.36 μM) expressed on cells involved in regulation of immune responses (Reddy et al. 1991). Art-E has revealed a very modest capacity to inhibit ADP-induced platelet aggregation *in vitro*, with an IC_{50} of 192 μM (Jantan et al. 2010). Later, Art-E was found to exert potent antiproliferative activities against different cancer cell lines, notably against breast adenocarcinoma MCF-7 and MDA-MB-231 cells (ED_{50} = 2.2 and 3.0 μg/ml, respectively) (Wang et al. 2004). It is slightly less active against another type of cancer cells derived from solid tumors, but also exerts a potent cytotoxic action against P-388 leukemia cells (Musthapa et al. 2009b). Art-E is the lead product in the series, with interesting anticancer and antimicrobial properties (see below). The anticancer effects have been characterized using different experimental models *in vitro* and *in vivo*. In particular, the compound has revealed a robust dose-dependent effect in a 4T1 breast carcinoma xenograft model. The oral administration of Art-E at 25, 50 and 100 mg/kg reduced drastically the growth of tumor in mice and reduced the appearance of metastasis, without any apparent toxicity (Etti et al. 2017a). The compound is well tolerated; it can be administered orally for up to consecutive 10 days at 30 mg/kg, without any significant effect on cholesterol, creatinine and blood urea nitrogen levels (Fukai et al. 2003).

Modest antibacterial effects have been reported with Art-E against *Escherichia coli* and *Bacillus subtilis* (Suhartati et al. 2008), and with Art-O against *B. subtilis* (Suhartati et al. 2016). Mild antibacterial effects have been noted with Art-E against *Pseudomonas aeruginosa* strain PA01 (MIC = of 32 μg/ml) whereas the compound was essentially inactive against the microorganisms *Providencia stuartii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* (Kuete et al. 2011). Art-E showed little activity against *S. aureus* strain ATCC25922 (MIC = 256 μg/ml) (Kuete et al. 2011) whereas a significant antibacterial effect has been reported with the strain ATCC 25923 (MIC = 3.9 μg/ml) and the methicillin-resistant strain ATCC BAA-1720 (MIC = 13.3 μg/ml) (Zajmi et al. 2015). A structural analysis using transmission electron microscopy revealed that Art-E disrupted the architecture of the bacterial cell, so as to facilitate the penetration of antibiotics (Zajmi et al. 2015). A similar type of effect on the bacterial cell wall has been reported with Art-I, despite it is structurally distinct from Art-E. Importantly, the membrane destabilizing effects of Art-E are reminiscent of those reported with morusin, which is able to disrupt the cell membrane architecture and inhibiting the phosphatidic acid biosynthesis pathway of *S. aureus* (Pang et al. 2019). In many ways, morusin and Art-E behave similarly.

The flavonoid Art-F displays little cytotoxic effects against cancer cells but has shown antimycobacterial activity against *Mycobacterium tuberculosis* (MIC = 6.25 μg/ml) (Namdaung et al. 2006). It has also revealed significant anti-plasmodial activity against the chloroquine-resistant strain FcB1, being only slightly less active than Art-B and more active than Art-A (IC_{50} = 4.9, 1.56, 2.20 μM for Art-A, B and F, respectively) (Bourjot et al. 2010). Art-F, isolated from *A. integer* var. silvestris Corner, has revealed a weak capacity to inhibit 15-lipoxygenase (Shaha et al. 2016). Art-F can be found in different *Artocarpus* species, including a hybrid between *A. heterophyllus* (jackfruit) and *A. integer* (champedak) which has been specifically developed in Thailand to produce tastier and larger fruits, and for disease resistance (Panthong et al. 2013).

The isolation and structural characterization of several artonins have been reported but no specific effect has been pointed out, such as with Art-G and -H (Hano et al. 1990b), Art-J, -K and -L (Aida et al. 1993), and Art-M, -N, O, -P (Hano et al. 1993). Art-O, found in *A. rigida*, has shown a modest anti-proliferative action against HT-29 human colon cancer cell (ED_{50} = 3.2 μM) (Ren et al. 2010).

Art-I is much more interesting because the compound has revealed activities against multidrug-resistant (MDR) *Staphylococcus aureus* strains. It is a complex molecule (Figure 4) initially isolated from *A. heterophyllus* (Hano et al. 1992) but also from the leaves of *Morus mesozygia* (Fozing et al. 2012). Art-I has been found to inhibit the enzyme phosphodiesterase I *in vitro* (IC_{50} = 15.4 μM) (Fozing et al. 2012) and to function as an efflux pump inhibitor and a generator of ROS in *S. aureus*. Interestingly, the compound revealed an unanticipated capacity to promote considerably the activity of antibiotics (reducing the MIC 500- to-1000 fold), via Art-I-induced cell membrane damages (Farooq et al. 2014). It could be a useful natural product to treat multi-drug resistant *Staphylococcus* infections. The total synthesis of these complex molecules has been reported recently, opening the door to the design of simpler, more active analogs (Liu et al. 2020).

Art-V has been first isolated from *A. altilis* (Hano et al. 1994). This plant, known as paparahua tree, is largely used as a traditional medicine in Amazonian Ecuador and other countries in Oceania and South America. Many flavones and terpenoids have been isolated from this plant (Luzuriaga-Quichimbo et al. 2019). Art-X is a rare compound, isolated from *A. heterophyllus*, analogous to the diarylheptanoid kuwanon R which has been characterized as an inhibitor of protein tyrosine phosphatase 1B (PTP1B) (Shinomiya et al. 1995; Hoang et al. 2009).

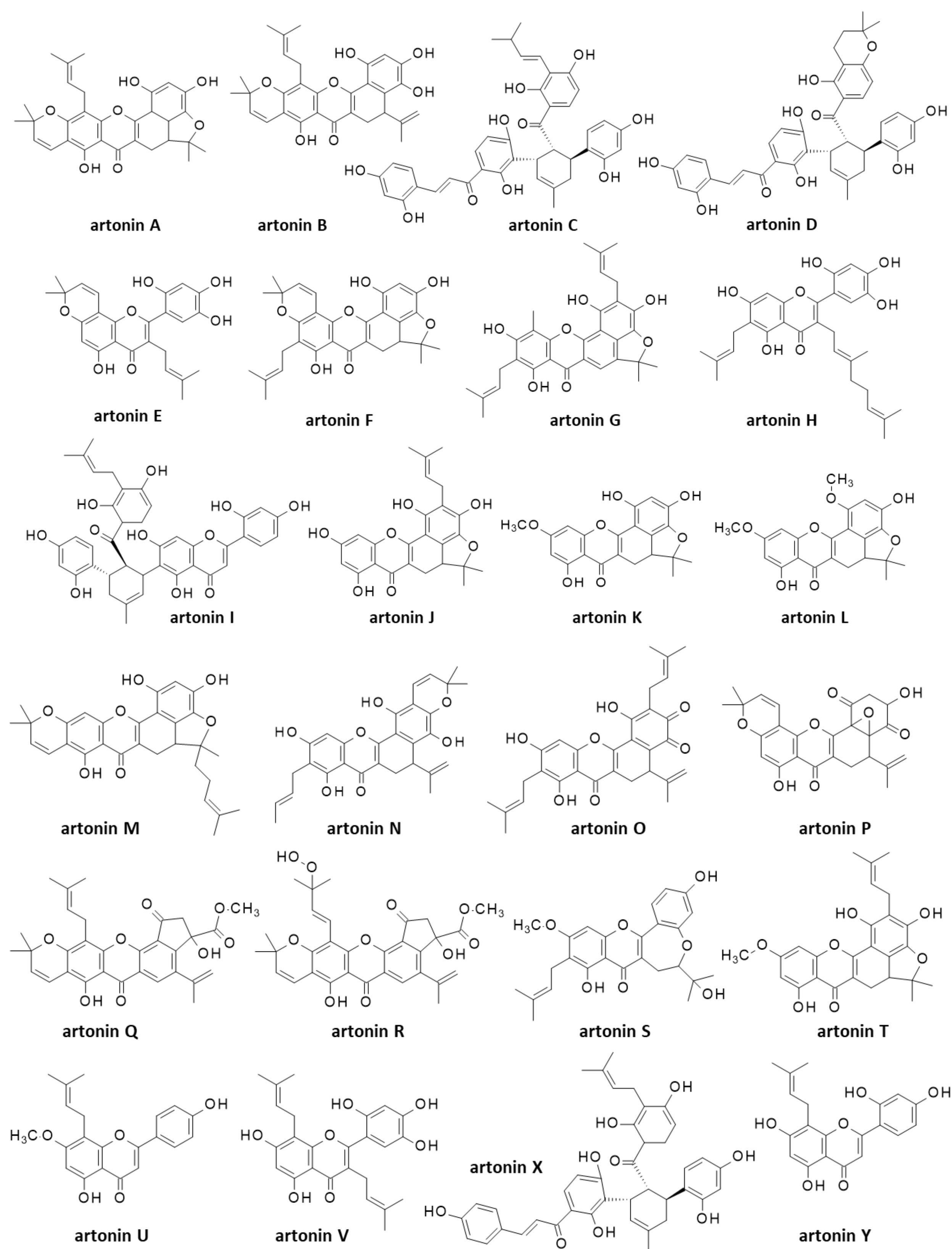

Figure 4. Chemical structures of artonins

ANTICANCER MECHANISM AND MOLECULAR TARGETS OF ARTONIN-E

The mechanism of action of Art-E has been thoroughly investigated, at least at the cellular level. The compound triggers a down-regulation of the anti-apoptotic protein MCL1 (myeloid leukemia cell sequence-1) in human A549 and H292 non-small cell lung cancer cells and sensitizes them to anoikis (detachment-induced apoptosis) (Wongpankam et al. 2012). In these NSCLC cells, Art-E down-regulates several proteins involved in cell migration and invasion, such as FAK (focal adhesion kinase) and CDC42 (Cell division cycle-42). At non-cytotoxic low concentrations (0.05-05 μg/ml), the compound markedly reduced the migration of H460 NSCLC cells *in vitro* (Plaibua et al. 2013). But the main characteristic of Art-E is certainly its capacity to overcome resistance of cancer cells induced by the ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). In gastric cancer cells, the effect is coupled with an Art-E-induced up-regulation of the death receptor 5 (DR5) and tumor suppressor p53 (Toume et al. 2015). A similar enhancement of TRAIL-induced apoptosis by Art-E has been reported recently with LoVo colorectal cancer cells. The compound down-regulated DR5 while it up-regulated the other major regulator of TRAIL-induced apoptosis called cFLIP (cellular FADD-like-IL-1beta-converting enzyme-inhibitory protein) on LoVo cells (Sophonnithiprasert et al. 2019). Remarkably, Art-E can trigger cell cycle arrest (S phase) and a massive mitochondria-dependent apoptosis in SKOV-3 ovarian cancer cells (Rahman et al. 2016) and in HCT116 colon cancer cells, Art-E induced apoptosis through upregulation of p-ERK1/2 (Nimnuan-ngam et al. 2020).

The capacity of Art-E to overcome TRAIL-resistance in cancer cells echoes the effects reported many years ago with Art-B and three other prenylated flavonoids isolated from *A. champeden*. Art-B and the structural analog heterophyllin were found to potently overcome TRAIL resistance in human gastric adenocarcinoma cells and to enhance the expression of DR5 (Minakawa et al. 2014). Many other bioactive natural products have been found to suppress TRAIL resistance, or to enhance TRAIL-induced apoptosis via the death receptor pathway (Dai et al 2015; Ahmed and Ishibashi 2016; Shahwar et al. 2019) but the potency of Art-E and Art-B is noticeably high. A similar capacity to induce TRAIL sensitization by regulating DR5 has been observed with morusin in glioblastoma cells (Park et al. 2016), suggesting thus a class effect. TRAIL emerges as a master element of the antitumor action of Art-E and its analogs (Figure 5).

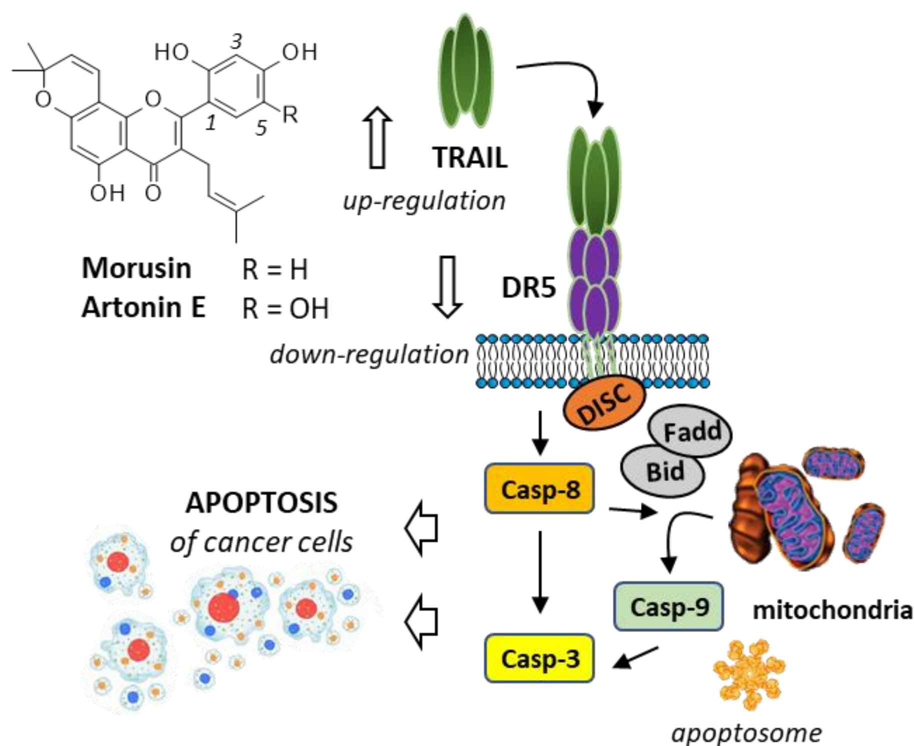

Figure 5. A schematic illustration of the anticancer mechanism of action common to morusine and artonin E (5-hydroxy-morusin). Both compounds trigger an up-regulation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and a down-regulation of the death receptor 5 (DR5). Upon trimerization of TRAIL and binding to DR5, the complex promotes the sequential recruitment of Fadd (Fas-associated protein with death domain) and caspase-8 (Casp-8) to form the death-inducing signaling complex (DISC). Processing of Bid (a BH3 domain-containing proapoptotic Bcl2 protein family member) by Casp-8 mediates mitochondrial damages. Activation of Casp-8 also directly induced Casp-3 activation, which concurs to the amplification of apoptosis.

Art-E has revealed marked activities against breast cancer cell lines. The compound inhibited the growth of estrogen receptor (ER) positive breast cancer cells MCF7 with an efficacy superior to that of the reference estrogen receptor modulator tamoxifen, at least *in vitro* (IC_{50} = 3.8 and 18.9 μM for Art-E and tamoxifen, at 72 hours, respectively). Molecular modeling predicted that Art-E can interact directly with the ligand-binding domain of hERα. The docking analysis indicated that Art-E can form more stable complexes with hERα compared to its analogs Art-U, -L, -S, and -T, and artoelastin (Etti et al. 2016). The analog isocyclomorusin is predicted to exhibit an even better binding capacity to hERα than Art-E (Fitriah et al. 2018). Art-E induces caspase-dependent apoptosis of MCF7 cells, associated with elevated production of ROS and an up-regulation of the cyclin-dependent protein kinase inhibitor p21 (Etti et al. 2017b). Similar results have been reported using MDA-MB 231 triple-negative breast cancer cells: up-regulation of p21, G2/M cell cycle arrest, and caspase-dependent apoptosis (Etti et al. 2017c). Binding of Art-E to hERα is entirely plausible but this computational hypothesis has not been validated experimentally. Other targets have been proposed for Art-E, based on *in silico* modeling, such as the inflammatory cytokine-induced ubiquitin-like modifier FAT10 which is known to target hundreds of proteins for degradation by the 26S proteasome. A molecular modeling analysis has predicted that Art-E could bind and inhibit FAT10 implicated in hepatic carcinoma, but here again, there is no experimental validation of this hypothesis (Chaturvedi 2015). In brief, from a mechanistic point of view, Art-E can be considered as a TRAIL regulator in cancer cells, but more work in needed to better evidence the upstream molecular targets. Proteins such as hERα and FAT10 may provide a receptor for the compound but more works are needed to validate these molecular modeling proposals.

DISCUSSION

Artocarpus species have been largely investigated to characterize their pharmacological properties, in line with the traditional medicinal uses of these plants. Thus far, most phytochemical studies have been centered around *A. altilis* and *A. heterophyllus* (Nomura et al. 1998; Sikarwar et al. 2014; Mohammed Haleel et al. 2018; Mainasara and Abu Bakar 2019; Buddhisuharto et al. 2021). The species *A. elasticus* has been less investigated, although this bark-fiber producing plant is used traditionally to treat inflammatory conditions, but also to design crafts, clothes, ropes, and building materials (Veriyan et al. 2019). The phytochemical analysis indicates that like its congeners, *A. elasticus* is rich in prenylated flavones, in particular compounds with a C-3 prenyl side chain, sometimes cyclized into a 5 or 6 membered ring, and occasionally with an additional prenyl chain on ring A or B. These types of C-prenylated flavonoids are frequent in *Artocarpus* species (Šmejkal et al. 2014; Molčanová et al. 2019).

The present analysis sheds light on the artonin group of prenylated compounds and points out the prenylflavone Art-E as an interesting anticancer natural product. Art-E displays a range of pharmacological activities, chiefly antioxidant and anti-inflammatory effects, similar to those reported with the analogous isoprene flavonoid morusin (Choi et al. 2020). It is a potent anticancer agent, inspiring for the design and development of novel anticancer agents. The structural analogy between morusin and Art-E deserves further attention. Morusin has been much more studied than Art-E as an anticancer agent but also for its antiviral and antidiabetic effects (Choi et al. 2020; Kim et al. 2021). The *in vitro* and *in vivo* anticancer effects of morusin have been extensively characterized and the compound appeared promising against cancer stem cells (Zoofishan et al. 2018). We have a lot to learn from morusin to better understand the mode of action of Art-E.

Art-E and related prenylated flavonoids represent interesting anticancer agents, potentially useful to design novel bioactive compounds. Promising data have been reported with morusin-loaded nanoparticles for the treatment of glioblastoma (Agarwal et al. 2019; Zheng et al. 2021) and with artocarpin (Tzeng et al. 2016). A similar potency could be anticipated with Art-E using nanoparticle delivery systems. This compound deserves further attention.

CONCLUDING REMARK

In conclusion, this review exposes the potential health benefit of *Artocarpus elasticus* Reinw. ex Blume used as a traditional medicine in Asia and presents for the first time the complete series of artonin bioactive natural products. An extensive analysis of the scientific information led us to underline the value of the lead compound artonin E for the design of novel anticancer agents. The anticancer properties of Art-E have been well-established, demonstrating its capacity to trigger apoptosis of different types of cancer cells, but additional research efforts are required to better characterize its molecular targets. Another takeaway message is that Art-E can be considered as a morusin derivative, endowed with similar, if not superior, anticancer properties. This isoprene flavonoid warrants further studies.

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Phytochemical analysis and in-vitro antimicrobial screening of the leaf extract of *Senna occidentalis* (Fabaceae)

A.A. TAMASI[1], M.O. SHOGE[1,♥], T.T. ADEGBOYEGA[2], E.C. CHUKWUMA[3]
[1]Department of Chemistry, Faculty of Science, Air Force Institute of Technology, PMB 2014, Nigerian Air Force Base, Kaduna, Kaduna State, Nigeria.
♥email: moshachemist@yahoo.com, m.shoge@afit.edu.ng
[2]Biology Unit, Faculty of Science, Air Force Institute of Technology, PMB 2014, Nigerian Air Force Base, Kaduna, Kaduna State, Nigeria
[3]Forest Herbarium Ibadan, Forestry Research Institute of Nigeria. Jericho Hills, Ibadan, Oyo State, Nigeria

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Abstract. *Tamasi AA, Shoge MO, Adegboyega TT, Chukwuma EC. 2021. Phytochemical analysis and in-vitro antimicrobial screening of the leaf extract of* Senna occidentalis *(Fabaceae). Asian J Nat Prod Biochem 19: 57-64.* Due to the use of *Senna occidentalis* as an antimalarial, anti-inflammatory, antioxidant, hepatoprotective, and antibacterial agent traditionally, this study examined the phytochemical composition and antimicrobial activity of the isolated methanol, ethyl acetate, and hexane fractions of the leaves of *Senna occidentalis*. Qualitative and quantitative phytochemical analyses were carried out using standard procedures. Antimicrobial activity was performed by using standard procedures against known common clinical isolates. Qualitative phytochemical analysis showed the abundant presence of saponins, tannins, flavonoids, and terpenoids in the ethyl acetate fraction. Cardiac glycosides were not detected in all the tested isolated fractions while steroids were found to be present and abundant in ethyl acetate and hexane fractions respectively. Quantitative phytochemical analysis showed that the methanol fraction contained 4%w/w alkaloids, 0.51%w/w flavonoids, 9.5%w/w saponins, 1.3%w/w tannins 5.5%w/w terpenoids, and 0.6%w/w total phenol content. The antimicrobial susceptibility test shows a range of inhibitory zone of 10-16 mm. Hexane fraction has the highest zone of inhibition against *Candida albicans*. The lowest MIC values of 0.6mg/mL and 0.2mg/mL were observed in ethyl acetate fraction against *Candida albicans* and *Escherichia coli* respectively. The highest MIC was 20mg/mL was observed in the ethyl acetate fraction against *Staphylococcus aureus*. The MMC values were varied widely. The MMC value against *Bacillus subtilis, Candida albicans,* and *Trichophyton rubrum* was 5mg/mL while the MMC value against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli* was 20mg/mL. The results from this study show that the leaves of *Senna occidentalis* can serve as a potential source of some phytochemicals and also have the potential to be developed as a source of antibiotics.

Keywords: Alternate medicine, antimicrobial, phytochemicals, *Senna occidentalis*

INTRODUCTION

Medicinal plants are traditionally used globally for the treatment of many diseases, including but not limited to; asthma, gastrointestinal problems, skin disorders, respiratory and urinary problems, hepatic and cardiovascular disease (Tian et al. 2014). Plants synthesize a wide range of biologically active compounds (Cushnie et al. 2014) that are important for them to survive and flourish in the natural environment, protecting them from abiotic stress, insect pests as well as disease infections (Vardhini and Anjum 2015). Since medicinal plants possess an effective source of antimicrobial natural products with proven potential of treating infectious diseases compared to the synthetic drug, there has been a gradual revival of interest in the use of medicinal plants in developed and developing countries (Akinyemi et al. 2006; Ajayi and Akintola 2010; Valarmathy et al. 2010). The natural products from medicinal plants are used in pharmaceutical preparations either as pure or as extracts (Gogtay et al. 2002).

Senna is a large genus of flowering plants in the legume family; Fabaceae (Leguminosae). This genus is distributed throughout the tropics comprising more than 300 species (Gebrelibanos et al. 2014). *Senna* species are commonly used as ornamentals, famine foods, and many species are used in both traditional and modern medicines (Haraguchi et al. 1998). *Senna occidentalis* (L.) Link. (Syn. *Cassia occidentalis* L.) is commonly known as coffee *Senna* (Odeja et al. 2014). It is found in open pastures and fields, cultivated with cereals such as soybean, corn, sorghum, and others (Barbosa-Ferreira et al. 2005). Various parts of *S. occidentalis* (seeds, roots, leaves, and stems) are traditionally claimed to be useful in treating different medical conditions and infections (Bekele and Reddy 2015). It has been used as a folklore medicine for hepatotoxicity treatment. The aqueous–ethanolic leaves extract was tested for hepatoprotective activity on liver damage in paracetamol-induced hepatotoxicity in rats shows that the paracetamol-induced hepatotoxic indications were to some degree reduced after 21 days of treatment with *Senna occidentalis* leaves extract in a dose-dependent manner (Uzzi and Grillo 2013). Aqueous extract of *Senna occidentalis* tested for antidiabetic activity showed a significant reduction in fasting blood glucose levels in the normal and alloxan-induced diabetic rats (Laxmi 2010).

Previous studies on *S. occidentalis* extract in four different solvents (methanol, aqueous, benzene, petroleum

ether, and chloroform) showed that methanol extract had antimicrobial activity against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, Staphylococcus aureus,* and *Staphylococcus epidermidis*. The aqueous extract was effective against *Proteus vulgaris, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa;* while benzene and petroleum ether extracts were active against *Proteus mirabilis* and *Escherichia coli;* and chloroform extract was inactive against all tested strains (Arya et al. 2010). Further, a study by Daniyan et al. (2011) showed that flower extract of *S. occidentalis* had maximum inhibition against *Klebsiella pneumoniae and* no activity against *Staphylococcus aureus, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa.* Thus, the flower extract of *S. occidentalis* can be used to treat *Klebsiella*-associated ailments such as pneumonia, bronchitis, and other diseases caused by *Klebsiella pneumonia.*

The antibacterial and antimalarial activities of leaves and root bark extracts of this plant have also been reported (Tona et al. 2001; Chukwujekwu et al. 2006). Studies on the ethanolic, dichloromethane and lyophilized aqueous extracts of *S. occidentalis* root bark were tested for antimalarial activity against *Plasmodium berghei.* The extracts produced significant chemo suppressions of parasitemia with 200 mg/kg dose when administered orally in experimental animals (mice). The species was found to be potential with 60% chemo suppression of the parasites.

Ibrahim et al. (2010) attempt to demonstrate the *in vitro* and *in vivo* activities of *S. occidentalis* against *Trypanosoma bruce.* Although the exact mechanism for the observed *in vivo* trypano-suppressive effect is not known, it is obvious that the extract contains some phytochemicals that could interfere with the survival of the parasites *in vivo*. The presence of alkaloids, flavonoids, tannins, and anthraquinones have been reported in this extract (Ogunkunle and Ladejobi 2006) and previous reports attributed the antitrypanosomal activity of several tropical plants to the flavonoids (azaanthraquinone), highly aromatic planar quaternary alkaloids, barbarine and harmaine (Nok 2001). The trypanosuppressive action of this extract could be due to the presence of one or more of these bioactive compounds. The present study examined the phytochemical compounds of *S. occidentalis* leaves which could be useful for the synthesis of orthodox drugs, and also its antimicrobial activity.

MATERIALS AND METHODS

Sample collection and preparation

The plant was collected at Unguwar Dosa, Kaduna, Nigeria and identified by Prof Gabriel Ajibade of Biological Sciences Department, Nigerian Defence Academy, Kaduna. It was authenticated at the Forest Herbarium Ibadan (FHI) (Holmgren et al. 1990). Upon collection, the leaves of the plant were rinsed with distilled water, air-dried at room temperature for two weeks, and then blended into fine powder. Active compounds from the plant were extracted in a crude form through cold maceration extraction method using methanol for 72 hours, concentrated at 40ºC using rotary evaporator and the crude extract fractionated with hexane, ethyl acetate, and methanol successively in a 500ml separating funnel.

Figure 1.0 illustrates the morphology of *S. occidentalis,* while Figure 2 shows the current distribution of the species in Nigeria.

Qualitative phytochemical screening

The hexane, ethyl acetate, and methanol fractions of *S. occidentalis* were assessed for the existence of some phytochemicals using the following standard methods:

Test for Alkaloids

(i) Dragendroff's reagent: 8 g of Bi ($NO_3.5H_2O$) was dissolved in 20 ml of nitric acid and 2.72g of potassium iodide in 50 ml of water, mixed and makeup to 100ml with distilled water.

Test: To 0.1g of extract, added 2.0 ml of HCl. To this acidic medium, 1 ml of reagent was added. An orange-red precipitate produced immediately indicates the presence of alkaloids.

(ii) Wagner's reagent: 1.0 g of iodine and 2.0 g of potassium iodide were dissolved in 5 ml sulphuric acid and the solution was diluted to 100 ml.

Test: 0.1g extract was acidified by adding 1.5% v/v HC1 and a few drops of Wagner's reagent. The formation of yellow or brown precipitate confirmed the presence of alkaloids.

(iii) Meyer's reagent:1.36 g Mercuric chloride was dissolved in 60 ml of distilled water and 5g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water.

Test: To 0.1g ml of extract, few drops of reagent were added. The formation of white or pale precipitate showed the presence of alkaloids.

Figure 1. Morphology of *Senna occidentalis* (L.) Link.

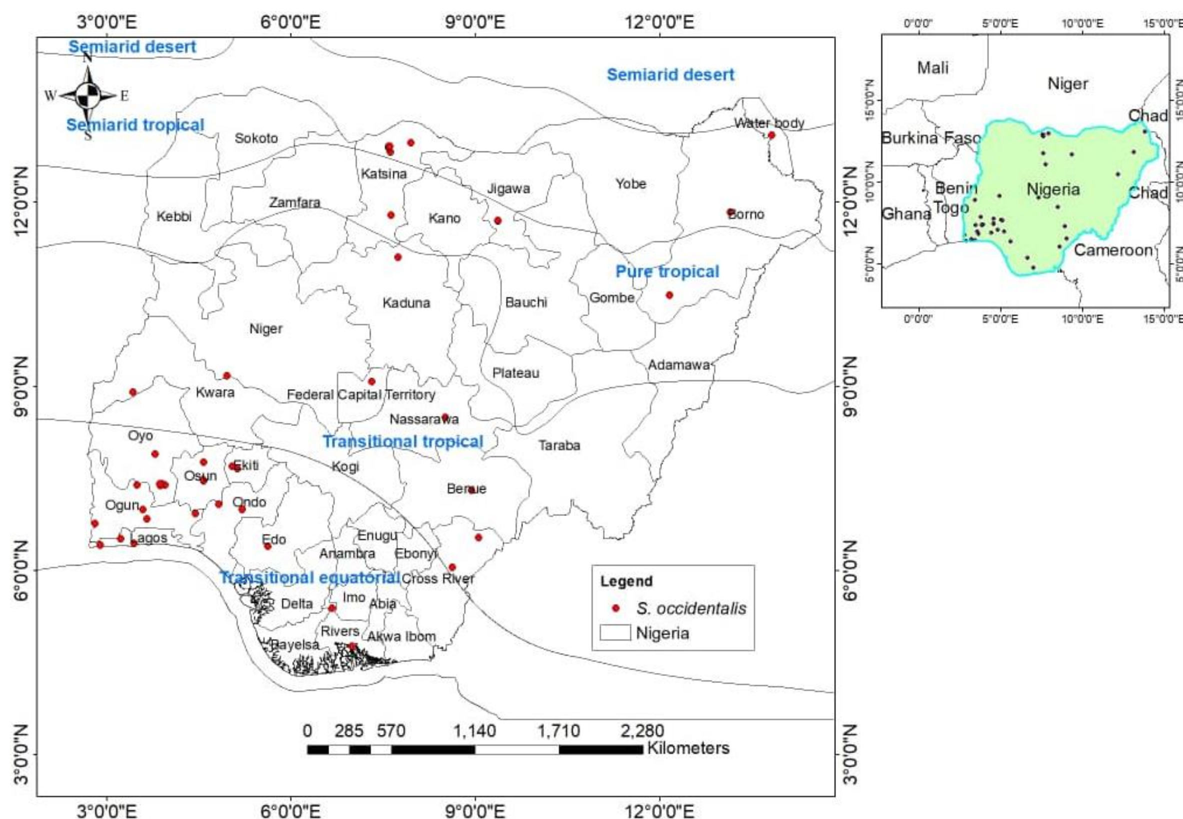

Figure 2. Distribution of *S. occidentalis* in Nigeria

Test for flavonoids: ammonia/H_2SO_4

The sample was added to 5 ml of diluted ammonia solution and concentrated H_2SO_4. The disappearance of yellow color on standing indicates the presence of flavonoids.

Test for saponins: Frothing Test

3 ml of the aqueous solution of the extract was mixed was stoppered in a test tube and shaken vigorously for about 30sec, then allowed to stand for 3min and observed for a persist honeycomb froth, which indicates the presence of saponins.

Test for tannins: Ferric chloride method

2 ml of the aqueous extract was added to 3 drops of 10% Ferric chloride solution. The occurrence of blue-black color showed the presence of tannins

Anthraquinone test: Borntrager's test

0.1g of the dried extract was placed in a dry test tube and 5 ml of chloroform was added and was heated in a steam bath for 1 min. The extract was filtered while hot and allowed to cool. A 10% ammonia solution was added to the filtrate and then shaken. The upper aqueous layer was observed for bright pink indicates the presence of anthraquinones.

Terpenoids test: Salkowski test

0.1g of the sample was mixed with 2ml of $CHCl_3$ in a test tube 3ml of concentrated H_2SO_4 was carefully added to the mixture to form a layer. An interface with a reddish-brown coloration was formed if terpenoids constituent is present.

Cardiac glycosides Test: Keller-Killiani test

A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ was mixed with 10 ml aqueous plant extract and 1 ml concentrated H_2SO_4. A brown ring formed between the layers showed the presence of cardiac glycosides.

Phenol test: Ferric chloride test

To 1 ml aliquot of each of the fractions, was added with 3-4 drops of neutral 5% ferric chloride solution. The formation of dark green color indicated the presence of phenols.

Steroids test: Liebermann-Burchard test

The extracts were added 5ml of chloroform, shake and filtered. and few drops of acetic anhydride along with a few drops of concentrated sulphuric acid were added through the side of the tube. The appearance of green color indicates the presence of sterols in the extracts.

Quantitative phytochemical screening

Quantitative determination of alkaloids

Quantitative determination of alkaloids was performed according to the phytochemical methods described by Harborne (1973). Exactly 200 cm^3 of 10% acetic acid in ethanol was added to each fraction (1g) in a 250 cm^3 beaker and allowed to stand for 4 hours. The fractions were concentrated on a water bath to ¼ of its original volume and 10ml of concentrated ammonium hydroxide was added until the precipitation was complete and filtered after 3 hours with a pre-weighed filter paper. The residues were dried in an oven and the percentage of alkaloid for each fraction is calculated mathematically as follows:

$$\% \text{ Alkaloids} = \frac{\text{Weight of alkaloid}}{\text{Weight of samples}} \times 100$$

Quantitative determination of terpenoids

Dried plant extracts (1g) of each fraction (Wi) were soaked in 20 ml of ethanol for 24 hours (Indumathi et al. 2014). After filtration, the extracts were extracted with 10 ml of petroleum ether using a separating funnel. The ether extract was separated into pre-weighed glass vials and kept completely dry (Wf). The yield (%) of total terpenoids contents was measured by the formula:

$$\% \text{ Terpenoids} = \frac{Wi - Wf}{Wi} \times 100$$

Quantitative determination of saponins

Quantitative determination of saponins was carried out using the method by Ejikeme et al. (2014). 100 cm^3 of 20% aqueous ethanol was added to 1g of each fraction in 250 cm^3 conical flasks. The mixtures were heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55°C. The residue of the mixtures was re-extracted with another 100 cm^3 of 20% aqueous ethanol after filtration and heated for 4 hours at a constant temperature of 55°C with constant stirring. The extracts were evaporated to 40 cm^3 over a water bath at 90°C. 20 cm^3 of diethyl ether was added to the concentrate in a 250 cm^3 separating funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. 40 cm^3 of n-butanol was added to the aqueous portion, shook, allowed to partition and the butanol layer collected after 10min. this process was also repeated twice and the combined butanol layer was washed with 20 cm^3 of 5% sodium chloride and heated in a pre-weighed crucible. The saponin content was calculated as follows:

$$\% \text{ Saponins} = \frac{\text{Weight of saponins}}{\text{Weight of samples}} \times 100$$

Quantitative determination of tannin (using the spectrophotometric method)

Tannins contents were quantitatively determined by the Folin-Ciocalteau method for ethyl acetate and methanol fractions. 0.1ml of each fraction were added to volumetric flasks (10 ml) both containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35 % Na_2CO_3 solution was diluted to 10 ml with distilled water. The mixtures were shaken well and kept at room temperature for 30 minutes. Reference standard solutions of gallic acid (32, 63, 125, 250, 500, and 1000 µg/mL) were prepared. Absorbance for test and standard solutions were measured against the blank at 760 nm with a UV/Visible spectrophotometer (Miean and Mohamed 2001; Ribarova and Atanassova 2005).

Determination of total flavonoid content

Total flavonoids content was determined from the calibration curve of quercetin and expressed as a microgram of quercetin equivalent per milligram of extract. Total flavonoid content was determined according to the procedure of (Chang et al. 2002), validated by (Nugroho et al. 2013) with some modifications. 1mg/1 ml standard solution was prepared, and series concentrations of quercetin were then obtained. The sample solution (0.5ml) was added with 1.5 ml methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml Potassium acetate 1M, and 2.8 ml of distilled water, and then incubated for 30 minutes. Absorbance was measured at 415 nm. Distilled water was used as blank. Total flavonoid content was expressed in mg quercetin per 1g of plant extract.

Total phenolic content

The total phenolic content of ethyl acetate and methanol extracts (200-1000 ug/mL) were quantified using the Folin -Ciocalteu's phenol reagent (Singleton and Rossi 1965) with gallic acid (50-200ug/mL) as standard. Each extract (1 ml) was added to 1 ml Folin-Ciocalteu 's reagent (diluted tenfold in distilled water) in separate test tubes. The content of each test tube was mixed and allowed to stand for five minutes at 25˚C in an incubator. 1 ml of 2 % sodium carbonate solution (Na_2CO_3) was added to the mixture. This was allowed to stand for 2 hours at 25˚C in an incubator and centrifuged at 1000 g for 10 minutes to get a clear solution. The absorbance of the supernatant was then determined at 760 nm using a UV spectrophotometer. Distilled water (1 ml) was added to 1 ml Folin-Ciocalteu's reagent (diluted ten-fold in distilled water) processed in the same way as done for the test samples and used as blank. All measurements were done in triplicates. The total phenolics were expressed as micrograms per milliliter of gallic acid equivalents through the calibration curve with gallic acid.

Antimicrobial susceptibility test of *Senna occidentalis*

Determination of antimicrobial activity by standard procedures

Isolates of micro-organisms were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria. The antimicrobial activity of methanol, ethyl acetate, and hexane fractions of *S. occidentalis* was determined against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Candida albicans,* and *Trichophyton rubrum.* A suspension of micro-organism pure culture by disk diffusion was spread evenly over the face of a sterile agar plate using a sterile swab. For all the fractions, 100 mg/mL and 50

mg/mL each were applied to the center of the various agar plates (in a fashion such that the antimicrobial doesn't spread out from the center). Gentamicin, 10ug/mL and 1% Ketoconazole were inoculated as controls. The agar plate was incubated for 24 hours at a temperature of 37°C. The zone of inhibition appears when the plant extract fractions exert a growth-inhibiting effect. The diameter of the inhibitory zone was measured in millimeters and it is related to the level of antimicrobial activity present in the fraction; the larger the inhibitory zone usually means the better the antimicrobial potency.

Determination of Minimum Inhibitory Concentration (MIC) & Minimum Microbicidal Concentration (MMC) (Pramila et al. 2012)

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of the compounds which inhibit the growth of the microorganisms. Minimal bactericidal concentration (MBC) is defined as the lowest concentration of the compounds that kill and show no growth of the microorganisms on an agar plate. The test microbes were grown in nutrient broth and potato dextrose broth for bacteria and fungus respectively as previously described and the cultures were adjusted to 0.5 McFarland standard turbidity. MIC and MBC values of the plant extract against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Candida albicans,* and *Trichophyton rubrum* were determined based on a micro-well dilution method. The 24-well microtiter plates were prepared by dispensing 2 ml of MH broth into each well and 3 µl of the bacterial and fungal inoculum. The plant extract was dissolved in the fractions to obtain the concentration of 1 mg/mL and was serially diluted and mixed thoroughly. Then the plates were incubated at 37°C for 24h

RESULTS AND DISCUSSION

The screening of plant parts for medicinal properties starts with crude extract preparation using a range of solvents with different polarities to dissolve different phytochemicals compounds in the plant parts. This is a vital step in drug development from the plant. Phytochemical constituents vary quantitatively and qualitatively in different plant species and even in plant parts of the same species depending upon various atmospheric conditions and storage conditions (Jahan et al. 2008). Three solvents (hexane, ethyl acetate, and methanol) were used for the extract preparation. The qualitative phytochemical screening of the three fractions is shown in Table 1.

Saponins and terpenoids were found to be present in all three extracts of *S. occidentalis.* Tannins, flavonoids, alkaloids, and phenols were found to be present in ethyl acetate and methanol extracts only. Steroids and anthraquinones were positive in hexane and ethyl acetate extracts, while cardiac glycoside was completely negative in all three extracts.

The occurrence of this wide range of phytochemicals shows the medicinal potentials of *S. occidentalis*. Alkaloids, saponins, tannins, flavonoids, and steroids have been known to be biologically active thus partially responsible for the antimicrobial activities of plants (Nethathe and Ndip 2011). Flavonoids are known to be synthesized by plants in response to microbial infection (Cowan 1999), antimicrobial properties of saponins are due to the leakage of proteins and certain enzymes from the cell (Zablotowicz et al. 1996) while tannins bind to proline-rich proteins and interfere with the protein synthesis (Shimada 2006).

As revealed in Table 2, the three fractions had a range of terpenoids from 5.5% to 7.5%w/w. Malik et al. (2017) also reported a wide range of terpenoid content, i.e. *Cassia absus* contains 40%, and *Carissa carandas* 80%, in which these two plants were used as medicinal plants in Punjab. Terpenoids are chemically interesting groups of secondary metabolites (Ajaib et al. 2016) of important therapeutic use as antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, antioxidants, and antiparasitic (Brahmkshatriya and Brahmkshatriya 2013). Jaeger and Cuny (2016) reported that terpenoids are naturally formed in a stereospecific manner and can be obtained in enantiopure form by extraction from natural sources. Thus, the pure enantiomeric form is needed for the production of highly active medical compounds of high pharmaceutical value.

Saponins have a wider range of 1.5% to 9.5% w/w across the tested fractions. A previous study by Ezeonu and Ejikeme (2016) also reported a wide range of saponin content between *Protea elliottii* (1.6%) to *Anogeissus leiocarpus* (12.5%). The extraction of saponins from these plants serves as a good source of saponins since saponins protect plants against microbial attacks such as yeast and fungal infections (Sheikh et al. 2013). Oyekunle et al. (2006) reported that saponins show antimicrobial activity by inhibiting the growth of Gram-positive and Gram-negative microorganisms. However, some saponins are not effective against Gram-negative microorganisms because saponins are not able to penetrate the cell membranes of Gram-negative bacteria.

Table 1. Phytochemicals compounds of three fractions of *S. occidentalis* leaves

Test	Hexane Fraction	Ethyl acetate Fraction	Methanol Fraction
Saponins	+ve	++ve	++ve
Tannins	-ve	++ve	+ve
Flavonoids	-ve	++ve	+ve
Cardiac glycosides	-ve	-ve	-ve
Anthraquinones	+ve	+ve	-ve
Terpenoids	+ve	++ve	+ve
Steroids	++ve	+ve	-ve
Alkaloids	-ve	+ve	+ve
Phenol	-ve	+ve	+ve

Note: ++ve: Abundant, +ve: Present, -ve: Absent

Table 2. Percentage chemical composition of *S. occidentalis* leaf fractions

Sample	% Alkaloids	% Flavonoid	% Saponin	% Tannin	% Terpenoid	% Total Phenol
Hexane fraction	0	0	1.5%w/w	0	6%w/w	0
Ethylacetate fraction	8%w/w	0.2%w/w	2.5%w/w	1%w/w	7.5%w/w	0.73%w/w
Methanol fraction	4%w/w	0.51%w/w	9.5%w/w	1.3%w/w	5.5%w/w	0.6%w/w

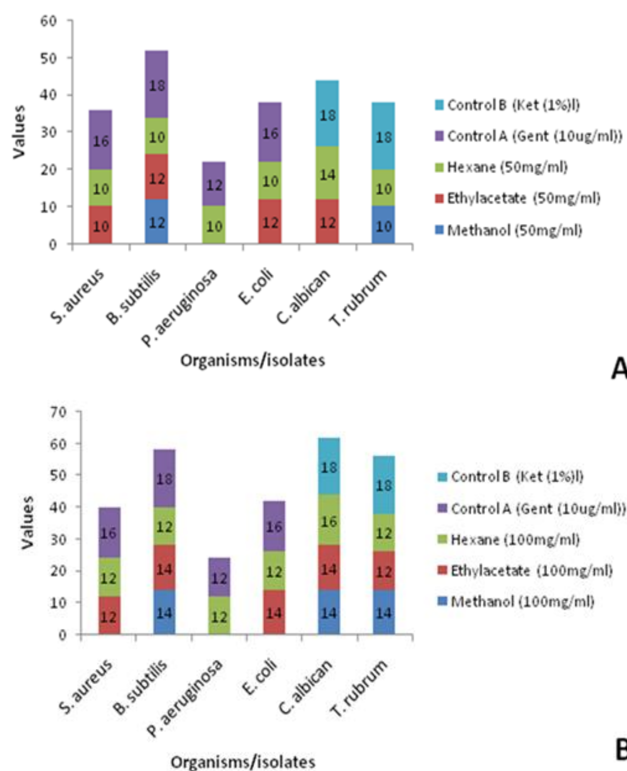

Figure 3. Diameter of inhibitory zone of *S. occidentalis* leaf fractions against several microbes. A. Fraction concentrations of 50 mg/mL; B. Fraction concentrations of 100 mg/mL

Flavonoids and phenols content in ethyl acetate and methanol were low, i.e. 0.2%w/w, 0.7%w/w, and 0.51%w/w, 0.6%w/w respectively. These results are also supported by previous studies on the flavonoid content of *Erythrina velutina* (0.2%) and *Mimosa tenuiflora* (0.21%) both of the plants that belong to the Fabaceae family with reported antimicrobial activity (Siqueira et al. 2012). Also, the low phenol content is similar to the report on stem bark (0.75%) and leaves (0.09%) of *Mangifera indica* (Okwu and Ezenagu 2008). Phenols protect plants from oxidative damage and do the same for humans (Okwu 2005).

Tannins content in ethyl acetate and methanol fractions were 1%w/w and 1.3%w/w respectively. Alkaloids were present in ethyl acetate fraction at 8%w/w and methanol fraction at 4%w/w. These values are much higher than reported values of alkaloids in *Cucumis sativus* (cucumber) and *Momordica charantia* (bitter melon) leaves with 1.23%w/v where bitter melon ethanolic leaf extract showed 70% percent inhibition against *E. coli, S. dysentariae 1,* and *S. pneumoniae* while *Cucumis sativus* leaf extract was found to have less antibacterial activity (Debnath et al. 2015). The differences in methanol and ethyl acetate differences could be due to their inherent polarity and hence influences their performance.

The antimicrobial activities of *S. occidentalis* against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Candida albicans,* and *Trichophyton rubrum* were examined in this study and their potency was qualitatively and quantitatively assessed by the presence or absence of inhibitory zones, minimum inhibitory concentration (MIC), and minimum microbicidal concentrations (MMC).

The zones of inhibition of the tested fractions against the selected bacterial and fungal strains are shown in Figure 3. in a concentration-dependent manner. The result showed that methanol and ethyl acetate fractions at 100 mg/mL and 50 mg/mL did not inhibit the growth of *Pseudomonas aeruginosa.* However, an inhibitory zone of 12 mm was observed for both hexane fraction and the tested control (Gentamicin) at 100 mg/mL and 10 µg/mL respectively. The zone produced by the standard antibiotic was similar to that produced by the hexane fraction of *S. occidentalis.* Similar to the present work, studies on the antibacterial activity of methanolic leaf extract of *Plukenetia conophora* against *Pseudomonas aeruginosa* isolated from urinary tract infection showed a concentration-dependent activity with *Pseudomonas aeruginosa* having zones of inhibition of 10, 12, 14, and 18 mm at 50, 100, 150 and 200 mg/mL respectively (Enitan et al. 2014).

The methanol fraction did not show any inhibition zone against *Staphylococcus aureus* and *Escherichia coli* at the concentrations of 100mg/mL and 50mg/mL. The diameter of inhibition of the ethyl acetate and hexane fractions at the concentration of 100mg/mL and 50mg/mL against *Staphylococcus aureus* was the same, i.e., 12mm and 10mm, respectively. These values were lower than positive control Gentamicin (16 mm). A study by Kumar et al. (2010) showed that the inhibitory zone of cardamom extract against *S. aureus* was 6 mm to 9 mm. The inhibitory zone of hexane, ethyl acetate, and dichloromethane extract against *Escherichia coli* was 12 mm.

The result showed a similar antibacterial activity of methanol and ethyl acetate fractions at the concentration of 100 mg/mL against *Bacillus subtilis* was 14mm, while hexane fraction has the inhibitory zone of 12mm. These values were lower compared to that of Gentamicin (18mm at 10ug/mL). The inhibitory zone of hexane fraction against *C. albicans* at the concentrations of 100 mg/mL and 50 mg/mL were 16mm and 14mm.

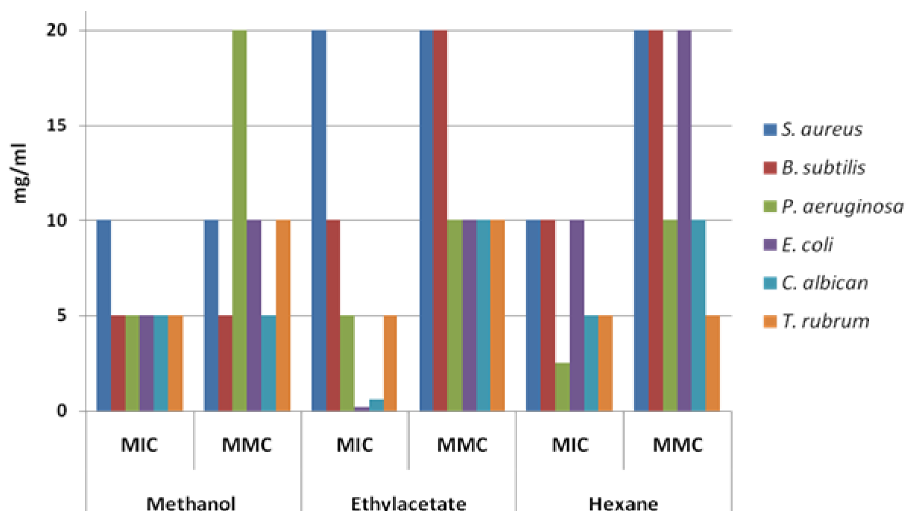

Figure 4. The Minimum Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC) of *S. occidentalis* leaf fractions

This fraction has a similar zone of inhibition for all tested bacteria at the concentrations of 100 mg/mL and 50 mg/mL, i.e. 12 mm and 10mm respectively. However, these values are much lower than the positive control Ket 1% (18 mm). Methanol extract of *S. occidentalis* showed a zone of inhibition of 14mm at 100mg/mL against *Trichophyton rubrum* and 12 mm for ethyl acetate and hexane fractions respectively, which are lower than that of positive control Ket 1% (18mm).

The MIC and MMC results of the tested fractions are depicted in Figure 4. Ethyl acetate fraction shows the lowest MIC value of 0.2mg/mL and 10 mg/mL MMC against *Escherichia coli*. This same fraction also has a MIC value of 0.6mg/mL and 10 mg/mL MMC against *Candida albicans*. The MIC and MMC values of hexane fractions were 2.5mg/mL and 10mg/mL against *Pseudomonas aeruginosa*. The MIC value (5 mg/mL) of methanol fraction was similar against all tested microbial strains except for *Staphylococcus aureus* with the MIC and MMC value of 10 mg/mL. The highest MIC value (20mg/mL) was recorded for ethyl acetate fraction against *Staphylococcus aureus*. Ethyl acetate fraction could be described as having a better antimicrobial activity than other fractions. The results of this study have added to the existing information on *S. occidentalis*, it is also important to consider its sustainability. On the distribution of *S. occidentalis,* the species seem to be more abundant around the transitional equatorial states in Nigeria (Oyo, Ogun, Lagos, Kwara, Osun, Edo, Ekiti, Ondo, Rivers, etc) and less in the Northern regions. Hence, its natural habitats need some attention, to ensure its sustainable collection and use.

In conclusion, the phytochemical analysis of the leaves of *Senna occidentalis* shows the presence of flavonoids, tannins, and phenols and the abundance of alkaloids, saponins, and terpenoids. Antimicrobial activities of the *S. occidentalis fractions* were more potent against the tested fungi; *Candida albicans* and *Trichophyton rubrum* than Gram-positive and Gram-negative bacteria. These results could be used as scientific evidence of the use of the plant as a source of remedy for various ailments traditionally. Further pharmacognostic studies are suggested to ascertain its potency in the treatment of diseases.

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Detection of terpenoids and steroids in *Lindsaea obtusa* with thin layer chromatography

YUSFIA URWATUL WUTSQA[1], SURATMAN[2,♥], SITI LUSI ARUM SARI[2]
[1]Department of Pharmacy, STIKes KHAS Kempek. Jl. Tunggal Pegagan, Kempek, Gempol, Cirebon 45161, West Java, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax. +62-271-663375, ♥email: suratman@staff.uns.ac.id

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Abstract. *Wutsqa YU, Suratman, Sari SA. 2021. Detection of terpenoids and steroids in* Lindsaea obtusa *with thin layer chromatography. Asian J Nat Prod Biochem 19: 65-69.* The diversity of ferns on this earth is very high, and the potential and benefits for human life are ornamental plants, food materials, and medicines. The benefits of not all ferns are known because of the lack of information on these ferns, one of the ferns is *Lindsaea obtusa* J. Sm. Ex Hook. The phytochemical test is the first step in research, searching for new active compounds derived from natural ingredients. This study was conducted to determine the differences of terpenoid and steroid compounds profile in *L. obtusa* extract using Thin Layer Chromatography (TLC). Extraction of *L. obtusa* using maceration techniques with methanol and n-hexane solvents. The extraction results will be used for phytochemical screening using the TLC technique with GF254 silica gel plate as a stationary phase, and a mobile phase is chloroform: n-hexane (9: 1 v/v), n-hexane: ethyl acetate (4: 1 v/v), and methanol: ethyl acetate (5: 1 v/v). Detection of terpenoid and steroids using Lieberman Burchard reagent. The result shows that *L. obtusa* extract contains terpenoid and steroid compounds. Quantitatively, the secondary metabolites of *L. obtusa* extract were soluble in methanol solvent more than the soluble compounds in n-hexane solvent. However, qualitatively, steroid compounds that dissolve in n-hexane solvents have a more varied Rf value than terpenoids, especially in the mobile phase of chloroform: n-hexane (9: 1 v/v) and n-hexane: ethyl acetate (4: 1 v/v). *L. obtusa* contains more various steroid compounds than terpenoid compounds.

Keywords: *Lindsaea obtusa*, phytochemistry screening, thin layer chromatography, terpenoids, steroids

INTRODUCTION

The diversity of ferns on this earth is very high. There are around 13,000 species of ferns on this earth, while the ferns in Indonesia, including the Malesiana area, are estimated to be 1250-1500 species (Machfira et al. 2016). Thus, the distribution of ferns is extensive, and the potential and benefits are high enough for human life, namely ornamental plants, foodstuffs, and medicines. However, the benefits of not all ferns are known because of the lack of information about the potentials of these ferns, one of which is the ferns *Lindsaea obtusa* J. Sm. Ex Hook.

Lindsaea obtusa is a terrestrial fern 20-30 cm tall. These ferns have root fibers with a diameter of 1-2.5 mm, brown. *L. obtusa* stem is hard, stiff, and black with a length of 4-25 cm. The leaves of *L. obtusa* are folium compositum pinnatus leaves and have black around petiolus. Leaflets are round or oval, asymmetrical at the tip and base, smooth and green in color. *L. obtusa* has a round, brownish-green sorus located on the underside of the leaf. On one leaf, there is approximately 10-13 sorus. *L. obtusa* grows in forest shrubs, especially in humid areas. This fern lives at around 1000 meters above sea level (Dong et al. 2016). The true ferns *L. obtusa* live in temperate Asian regions such as Taiwan and tropical climates such as Indonesia, Java, and Bali.

The phytochemical test is an essential first step in research searching for new active compounds derived from natural ingredients. This information can be used as initial information to synthesize new drugs or to become prototypes of certain active compounds (Kruk et al. 2021). Terpenoids are also called isoprenoids, this is because the carbon skeleton is the same as the isoprene compound. Synthesis and accumulation of terpenoid compounds contribute to medicinal properties for treating respiratory inflammation, atopic dermatitis, arthritis, and neuroinflammation among various inflammatory diseases (Kim et al. 2020).

Steroids are lipids that do not have fatty acid groups and are not ester derivatives. Steroids are complex, fat-soluble organic molecules. This compound acts as a significant component of cell tissue (Rashidinejad et al. 2021). Synthetic steroids are glucocortico-steroids, estrogen, methylprednisolone, corticosteroids, androgens, squalamine, and hydrocortisone. These steroid compounds commonly treat diseases due to neurodegenerative disorders (Bansal and Singh 2018). Ferns from the Equisetaceae, Osmundaceae, Lygodiaceae, Dennstaedtiaceae, Woodsidaceae, Thelypteridaceae, and Dryopteridaceae families also contain steroid compounds (Yokota et al. 2017).

The method that can be used for phytochemical screening is chromatography. Chromatography is the separation of a mixture of compounds in a sample based on differences in the interaction of the sample with the stationary and mobile phases. One of the chromatography methods used for phytochemical screening is Thin Layer Chromatography (TLC). Separation in Thin Layer

Chromatography is based on differences in polarity between the sample and the solvent. The stationary phase is a solid that is applied flat in glass or aluminum as support. The mobile phase is a mixture of several liquids of different polarities. Research on the content of terpenoids and steroids in *L. obtusa* was minimal. Therefore this study was conducted to determine the difference of terpenoids and steroid profile in *L. obtusa* J. Sm. Ex Hook.

MATERIALS AND METHODS

Sample preparation

A sampling of *L. obtusa* was carried out using the cruise method, namely exploring the KGPAA Mangkunagoro I Botanical Forest Park, Ngargoyoso, Karanganyar, Central Java, Indonesia, which generally contained these ferns. The extraction process and phytochemistry screening was conducted in the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia.

The samples of *L. obtusa* ferns were separated from dirt or foreign materials and then cleaned with running water until clean. Next, this sample dried until the fern had reduced its moisture content. After drying, the sample is mashed using a blender until it becomes a powder and then weighed, then used for the extraction process (Seremet et al. 2020).

Secondary metabolites extraction

The extraction of secondary metabolites was carried out by the maceration method using methanol and n-hexane as solvents. 100 g of *L. obtusa* powder was macerated using methanol as a solvent with a ratio of 1 : 5, which was carried out overnight. The same is done with the n-hexane solvent. The extraction process is carried out by soaking in a solvent and then filtered using filter paper. The solvent is evaporated with a rotary evaporator at 400C, a speed of 40 rpm, and a pressure of 0.06-0.08 MPa until a thick extract is obtained for approximately 1 hour (Sati et al. 2019). The viscous extract is weighed, and the yield is calculated against the weight of the initial simplicity. The formula for the percentage of extract yield according to Wahyuni and Widjanarko (2015) is :

$$\text{Yield Extract} = \frac{\text{extract weight obtained (g)}}{\text{extracted weight (g)}} \times 100\%$$

Thin layer chromatography (TLC) profile analysis

Chemical compound profile analysis was performed on each extract using the Thin Layer Chromatography (TLC) method. First, the n-hexane and methanol extracts were eluted using the same stationery and mobile phases. This was done to determine the results indicated by different color spots on TLC for each extract (Rubiyanto 2013). Then, the n-hexane and methanol extracts were spotted on the TLC plate with the silica gel GF254 as stationary phase and eluted using the mobile phase in the form of chloroform: n-hexane (9:1 v/v), n-hexane: ethyl acetate (4:1 v/v), and methanol: ethyl acetate (5:1 v/v) in the developer's vessel. GF254 silica gel plate was made with a width of 2 cm and a length of 9 cm and given an initial limit of 1 cm and an end limit of 0.5 cm. The limit of the solvent is below the line where the spots are. After the eluent reaches the finish line of elution, the plates are removed and dried.

Analysis of secondary metabolite compounds

Lieberman Burchard spray reagent used the detection of terpenoid and steroid content from n-hexane extract and methanol extract. The terpenoid and steroid compounds observed the parameters from *L. obtusa* and the Rf (Retardation Factor) value (Yin et al. 2017). In thin-layer chromatography, the degree of retention is expressed as Rf, which can be formulated:

$$\text{Retardation Factor (Rf)} = \frac{\text{Distance of movement of the solute}}{\text{Distance of movement of the solvent}}$$

Lieberman Burchard is a spotting reagent for detecting steroids and terpenoids. The Lieberman Burchard spray reagent was prepared by mixing 5 ml of acetic acid anhydride with 5 ml of concentrated sulfuric acid, then adding this mixture to 50 ml of absolute ethanol. Each substance mixing was carried out by cooling. The application of this method to the TLC plate was sprayed with Lieberman Burchard reagent and then heated for 10 minutes at a temperature of 1000C. The presence of terpenoids is indicated by the appearance of a red-violet color, while the presence of steroids is indicated by the formation of a reddish-brown color (Gummadi et al. 2021).

RESULTS AND DISCUSSION

Secondary metabolite extraction

Extraction of secondary metabolites from *L. obtusa* was carried out by maceration method using methanol and n-hexane as solvents. The results showed that the extraction with methanol as a solvent produced a higher yield than n-hexane (Table 1).

Extraction with methanol solvent resulted in 5.8 g, so that the yield percentage was 5.8%. The extraction result with n-hexane solvent resulted in 0.7 g, so that the yield percentage was 0.7%. The difference in yield percentage is due to differences in the solubility of the extracted compound in each solvent used to affect the yield and characteristics of the extracted chemical compound. The n-hexane solvent is intended to dissolve semi-polar to nonpolar compounds, while methanol is used to dissolve more polar compounds. Compounds can be said to be nonpolar compounds if they have bonds between atoms with an attractive ability to gain electrons together, resulting in a distant state where the electrons are shared equally. Meanwhile, if a compound has one atom capable of attracting electrons stronger than other atoms and the electrons from the bond will not be used together equally, then the compound is called a polar compound (Tunega et al. 2020). These results are similar to those of (Azka and

Abdullah 2012), who showed that the secondary metabolites of water clover ferns *Marsilea crenata* most of them also dissolved in polar solvents with the yield of methanol extract of 11.98%, ethyl acetate extract of 1.37%, and chloroform extract of 0.31%. Thus, methanol is a polar solvent, while ethyl acetate and chloroform are semi-polar and nonpolar solvents.

Thin layer chromatography profile analysis

Research shows that *L. obtusa* extract contains terpenoid and steroid compounds. This is indicated by a positive reaction to the Liebermann-Burchard reagent (Table 2). The Liebermann-Burchard response was first described by Liebermann and then extensively developed by Burchard (Xiong et al. 2007). The Liebermann-Burchard reaction has been studied to determine the sterol content. Libermann Burchard reagent produces a varied color response depending on the double bond system, other functional groups, and the presence of nonpolar bonds (Xiong et al. 2002). Thus, Liebermann-Burchard is a spotting reagent for detecting steroids and terpenoids. This analysis was based on the ability of terpenoid and steroid compounds to form color with concentrated H2SO4 in anhydride acetic acid (Parbuntari et al. 2018). Therefore, the Liebermann-Burchard reagent will show a red-violet stain which indicates the presence of terpenoid compounds. In contrast, the presence of steroids is indicated by the appearance of a reddish-brown stain.

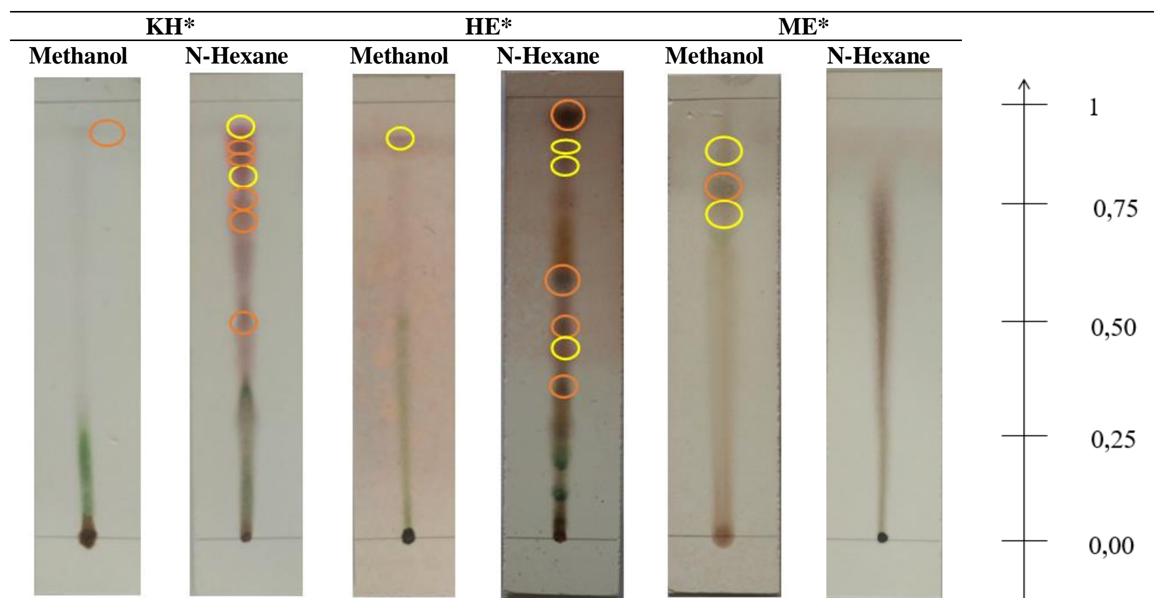
Two fractions give negative results on the detection of terpenoids with no visible red-violet spots, namely in the mobile phase methanol extract of chloroform: n-hexane (9: 1 v/v) and n-hexane extract in the mobile phase of methanol: ethyl acetate (5 : 1 v/v) (Table 2). In the detection of steroids, two fractions gave negative results with no visible reddish-brown spots, namely the mobile phase methanol extract n-hexane: ethyl acetate (4: 1 v/v) n-hexane extract in the mobile phase of methanol: ethyl. acetate (5: 1 v/v).

Table 3 shows that the Rf values for detecting terpenoid compounds in the mobile phase of chloroform: n-hexane (9: 1 v/v) n-hexane extract are found at Rf 0.82 0.93. The methanol extract in the mobile phase of n-hexane: ethyl acetate (4: 1 v/v) was found at Rf 0.90 and for n-hexane extract 0.42; 0.76; and 0.85. Methanol extract in the mobile phase of methanol: ethyl acetate (5: 1 v/v) was found at Rf 0.73 and 0.88. Meanwhile, the Rf value for the detection of steroid compounds in the mobile phase of chloroform: n-hexane (9: 1 v/v) methanol extract was found at Rf 0.93 and for n-hexane extract 0.49; 0.64; 0.73; 0.86; and 0.90. The n-hexane extract in the mobile phase of n-hexane: ethyl acetate (4: 1 v/v) was found at Rf 0.34; 0.48; 0.58; and 0.96. Methanol extract in the mobile phase of methanol: ethyl acetate (5: 1 v/v) was found at Rf 0.78. These results indicate that the n-hexane extract gave clearer results than the methanol extract, especially in the mobile phase of n-hexane: ethyl acetate (4: 1 v/v).

Tabel 1. Maceration result of *Lindsaea obtusa* extract

Solvent	Powder mass (g)	Extract mass (g)	Yield (%)
Methanol	100	5.8	5.8
N-hexana	100	0.7	0.7

Table 2. Thin Layer Chromatography Profile (TLC) of *Lindsaea obtusa* extract with Liebermann-Burchard reagent



Note: *KH = chloroform: n-hexane (9: 1 v/v); HE = n-hexane: ethyl acetate (4: 1 v/v); ME = methanol: ethyl acetate (5: 1 v/v). The yellow sign indicates the presence of a terpenoid compound, and the orange sign indicates the presence of a steroid compound

Table 3. Retardation factor (Rf) value of *Lindsaea obtusa* extract with Liebermann-Burchard reagent

Senyawa	KH		HE		ME	
	Methanol	N-Hexane	Methanol	N- Hexane	Methanol	N- Hexane
Terpenoid	-	0.82	0.9	0.42	0.73	-
		0.92		0.76	0.88	
				0.85		
Steroid	0.93	0.49	-	0.34	0.78	-
		0.64		0.48		
		0.73		0.58		
		0.86		0.96		
		0.9				

Note: *KH = chloroform: n-hexane (9: 1 v/v); HE = n-hexane: ethyl acetate (4: 1 v/v); ME = methanol: ethyl acetate (5: 1 v/v).

This is also found in medicinal Herb *Hypochaeris radiata* using the High-Performance Thin Layer Chromatography (HPTLC) technique (Senguttuvan and Subramaniam 2016). The thin layer chromatography profile results showed that red-violet spots were more visible in the n-hexane extract than in the methanol extract. This is consistent with the nonpolar secondary metabolites of terpenoids and steroids. Most of the terpenoid compounds also contain an –OH group so that the presence of a hydroxyl group substituent attached to the hydrocarbon chain can be attracted by semi-polar and even polar solvents (Stachowiak et al. 2020). So it can be concluded that *L. obtusa* positively contains secondary metabolites in the form of terpenoids and steroids. Other ferns, such as *Azolla microphylla*, also contain terpenoid and steroid compounds (Rashad 2021).

In conclusion, *Lindsaea obtusa* J. Sm. ex Hook extract contains terpenoid and steroid compounds. Quantitatively, the secondary metabolites of *L. obtusa* extract that were soluble in methanol solvent were more than the soluble compounds in n-hexane. However, qualitatively, steroid compounds that dissolve in n-hexane solvents have a more varied Rf value than terpenoids, especially in the mobile phase of chloroform: n-hexane (9: 1 v/v) and n-hexane: ethyl acetate (4: 1 v/v).

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Physicochemical composition of some new craft beers consumed in Maroua town from Far North region of Cameroon

M. DIGUIR[1], A. LAYA[2,3,♥], H. WANGSO[1], J.P. BAYANG[2], B.B. KOUBALA[1,3]
[1]Department of Chemistry, Faculty of Science, University of Maroua. P.O. Box 814 Maroua, Cameroon
[2]Department of Biological Sciences, Faculty of Science, University of Maroua. P.O. Box 814 Maroua, Cameroon
[3]Department of Life and Earth Sciences, Higher Teacher's Training College of Maroua, University of Maroua. P.O. Box 55 Maroua, Cameroon
[♥]email: laya.alphonse@yahoo.fr

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Abstract. *Diguir M, Laya A, Wangso H, Bayang JP, Koubala BB. 2021. Physicochemical composition of some new craft beers consumed in Maroua town from Far North region of Cameroon. Asian J Nat Prod Biochem 19: 70-80.* The work aimed to investigate the physicochemical composition of various craft beers (CB) and their distillates produced in Maroua town. The beers named *"Bil-Bil", "Cochette"* and *"Furdu"* were collected both morning and evening time. Dry matter, pH, titratable acid, total dissolved solids, proteins, sugars, carbohydrates, amino acids and alcohol content were evaluated. The results showed that local beers had a pH ranged from 4.06 to 4.22, 4.15 to 3.84 and 3.53 to 3.37 for "*Furdu*", "*Cochette*" and "*Bil-bil*", respectively. The alcohol varied between 5.40 and 6.92%, 1.08 and 5.10%, 1.73 and 2.88 for "*Furdu*", "*Cochette*" and "*Bil-bil*", respectively. The sugars ranged from 11.85 to 19.05 mg/mL for "*Furdu*", from 2.20 to 9.46 mg/mL for "*Cochette*" and from 9.92 to 19.78 mg/mL for "*Bil-bil*". For amino acids, the values varied from 3.40 to 7.37 mg /mL for "*Furdu*", 3.98 to 6.01 mg / mL for "*Cochette*" and 2.95 to 3.24 mg/mL for "*Bil-Bil*". Regarding protein, the values ranged from 0.80 to 0.85 mg/mL for these three CB. The distillates of CB collected evening showed high alcohol in fraction 1. Thus, these CB analysed can be promoted for alternative beer in Maroua town. Furthermore, the unsold CB can be distilled into ethanol.

Keywords: Alcohol, craft beers, distillate, Maroua town, physicochemical

INTRODUCTION

In Africa, cereals are the most widely cultivated and consumed as a staple food in many African households. These cereals are also used to prepare many fermented by-products. A local transformation unit of these cereals has been found in many areas of Cameroon, Republic of Central Africa and Chad (Djanan et al. 2002). The enterprises are mostly performed by women who recruit at least three workers (Maoura et al. 2002). In Cameroon, especially in the Northern part of Cameroon, various cereals such as sorghum, maize, yellow millet, *S35*, rice and fonio are processed into alcoholic and non-alcoholic beverages called locally beer or ancestor drink. These local beers produced are well appreciated by numerous people of Cameroon and other countries because these are a good source of nutrients such amino acids, carbohydrates, vitamins, minerals, phenolics, etc. (Maoura et al. 1999; Bamforth 2002; Lyumugabe et al. 2012; Cirimina et al. 2018). Because of their probiotics these local beers as known as therapeutic agents. The local beer is a beverage of significant historical and cultural importance. It is gaining popularity of craft beer and is of growing interest in several countries. It is also providing alternatives to mainstream beer production (Einfalt 2021). To date, craft beer is one of the fastest-growing alcoholic beverage industries throughout the world (Gómez-Corona et al. 2016).

These local beers are known as *Bil-Bil Furdu* and *Cochette,*the most widely produced and consumed. According to the tribes, these beers have different name, "*Bil-Bil*" beer is known as "Muzum" in Guiziga; "Ouzomm" in Mada, "Zoum" in Mafa; "Balda" in Guidar; "Himi" in Moudang; "Yii" in Toupouri; "Tcheu" in Kapsiki "Mbolo" in Fali; "Mgba" in Laka; "Amgba" in Baya; "Do'di" in Dourou and "Coumouille" in Kera. However, "*Furdu*" beer is known as "Bazdltah" in Mada, "Pram" or "Bazla Babara" in Guiziga, "Mpedli" in Kapsiki. The "*Cochette*" beer is known as "*Cochette*" in almost all tribes but differs by the pronunciation except the name in Mada tribe, "*Mohosso*". The local name of *Bil-Bil* vary from one country to another as *Bili-Bili* in Cameroon and Chad, *Dolo* in Burkina-Faso, Mali and Senegal; *Tchapalo* in Ivory Coast, *Tchoukoutou* in Benin, *Pito* in Ghana, and *Impéké* in Burundi (Odunfa 1985; N'da et al. 1996; Kayodé et al. 2005). While, *Furdu* and *Cochette* are new local beers which are not documented in the literature. The energy is provided at 70% by alcohol and 30% by carbohydrates (Lariven and Rigal 2017). According to Djanan et al. (2002), one litre of *Bil-Bil* contributes significantly to the recovery of iron magnesium, manganese, phosphorus and calcium dietary allowance per day for an adult. Various studies of *Bil-Bil* beer are investigated, however, these parameters are absent for *Furdu* and *Cochette* beers. Setta et al. (2020) reported that traditional African fermented cereal-based beverages are potential probiotic carriers because of the probiotic *Lactobacillus* spp. and yeasts which are involved in the

fermentation of such products, and can be used as probiotic health benefits to the majority of African populations.

The main concerns of all the local beers is their consumption for one to two days and the next day, these beers become more acidic due to the breakdown of ethanol into acetic acid (oxidation reactions). Finally, the unsold local beers are usually thrown. These unsold beers can be used in the production of quality by-products in order to have added value and solve pollution problem.

Thus, the present study was aimed to determine the physicochemical composition and distil some craft beers named *Bil-Bil*, *Furdu* and *Cochette* in order to valorize the local beers mostly consumed in Maroua town of Cameroon.

MATERIALS AND METHODS

Survey

The survey was conducted in order to find out more about the micro-enterprises in charge of the production of local beer in the town of Maroua, region of the Far North of Cameroon. A preliminary survey was carried out in all streets of the city of Maroua. We identified four streets where they produced mostly local beers such as Pitoare, Pont-vert, Ouro-tchede and Palar. During this survey, several types of local beers are consumed in the town of Maroua. However, on the basis of the most consumed in the city, we have identified three (03) types of local beers: *Furdu*, *Cochette* and *Bil-Bil*. After this first step, we conducted a survey using direct interviews with the producers and consumers of these beers. We interviewed 150 producers and 200 consumers of *Bil-Bil* beer, 20 producers and 35 consumers of *Frudu* beer and 20 producers and 40 consumers of *Cochette* beer.

Samples

Samples of *Cochette*, *Furdu* and *Bil-Bil* beers were purchased through the various producers located in four streets (Pitoare, Pont-vert, Ouro-tchede and Palar). The collected samples in the morning (start of the sale) and in the evening (end of the sale) from the suppliers and were introduced in suitable and sterile container and then labelled (Figure 1). Samples were transported to the Biochemistry Laboratory and Biological Chemistry of the University of Maroua (LabBBC). All samples were kept at-10°C before physico-chemical analyses and distillation.

Processing flow charity of different beers preparation

All the craft beers were produced by using different cereals shown in Figure 2.

Shortly, the cereal was soaked for 24h and washed prior for germination (2-3days). The germinated cereal was then sun-dried before milling and the malt flour was mixed with water and the mash mixture was decanted for 30min. The supernatant was heated for 2-3hr and its allowed to rest for overnight. Then, the second heating was done for 2-4 to obtain the sweet wort which was cooled for 3hr or more. Finally, the wet yeast was added and fermented overnight to obtain *bil-bil beer*. Figure 2.A shows the *Bil-Bil* beer preparation.

In brief, 7.0 kg of cereal was soaked for 1-2 days before sundering for 3hrs and milling in order to obtain flour (malt). Then, mixed with water before boiling at 95°C and the mash obtained was cooled at room temperature for 3hr before adding the wet yeast. The mixture was fermented for 10-12hrs and then filtered throughout the traditional filter tissue. The filtrate was poured in jar and named *Cochette beer*. The Figure 2.B shows different steps to produce *Cochette* beer.

Briefly, 3-7kg of cereal was wetted, cleaned or watered and germinated for three days and sun-dried before milling and the malt flour obtained was mixed with water, heated (95°C) and the mash obtained was cooled at room temperature for 4hr. Then, the mash was fermented for 72-100hr and then filtered throughout the traditional filter mesh. The product obtained was poured in jar and the *furdu beer* was ready for drinking. Figure 2.C is showing the summary methodology to produce *Frudu* beer.

A B C

Figure 1. Sample of different craft beers mostly produced and consumed in Maroua town, Cameroon. A. Bil-bil Beer, B. Cochette Beer, C. Furdu Beer

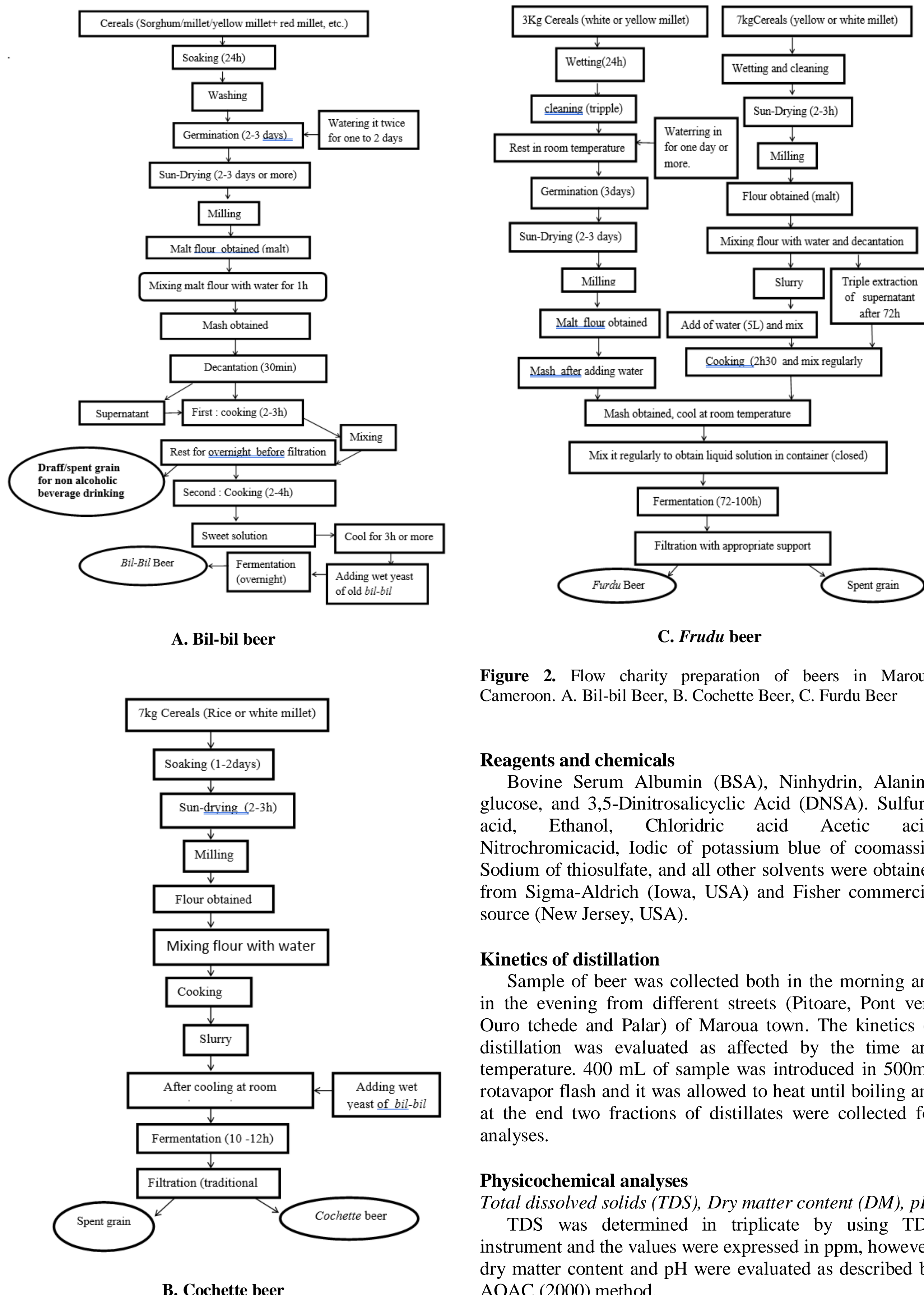

Figure 2. Flow charity preparation of beers in Maroua, Cameroon. A. Bil-bil Beer, B. Cochette Beer, C. Furdu Beer

Reagents and chemicals

Bovine Serum Albumin (BSA), Ninhydrin, Alanine, glucose, and 3,5-Dinitrosalicyclic Acid (DNSA). Sulfuric acid, Ethanol, Chloridric acid Acetic acid, Nitrochromicacid, Iodic of potassium blue of coomassie, Sodium of thiosulfate, and all other solvents were obtained from Sigma-Aldrich (Iowa, USA) and Fisher commercial source (New Jersey, USA).

Kinetics of distillation

Sample of beer was collected both in the morning and in the evening from different streets (Pitoare, Pont vert, Ouro tchede and Palar) of Maroua town. The kinetics of distillation was evaluated as affected by the time and temperature. 400 mL of sample was introduced in 500mL rotavapor flash and it was allowed to heat until boiling and at the end two fractions of distillates were collected for analyses.

Physicochemical analyses

Total dissolved solids (TDS), Dry matter content (DM), pH

TDS was determined in triplicate by using TDS instrument and the values were expressed in ppm, however, dry matter content and pH were evaluated as described by AOAC (2000) method.

Titratable acidity

Titratable acidity was determined as described by Ranganna (1979). The values were expressed in milligram equivalent oxalic acid per 100 grams of sample beer.

Evaluation of reducing sugars content

Reducing sugars content was determined using the dinitrosalicylic acid as described by Miller (1972). The results were expressed in mg per mL of sample beer.

Evaluation of total carbohydrates content

Total carbohydrates content was determined as described by Dubois et al. (1956). The results were expressed in mg per mL of sample beer.

Determination of total amino acids, proteins and alcohol content

Total amino acids content was determined using ninhydrin (1 %) reagent as described by Michel and Hannequart (1968). The results were expressed in mg of amino acids per mL of sample beer.

Total soluble proteins content was determined using Bradford (1976) method. The results were expressed in mg of BSA per mL of beer sample.

The content of alcohol in sample beer was determined as described by Caputi et al. (1968).

Statistical analyses

SPSS 20.0 Statistical Package for Windows (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analysis. Experiments were done in triplicate and one-way analysis of variance (ANOVA) was performed. Tukey's (HSD) test was used to determine any significant difference between different beers and the significance was accepted at level $p < 0.05$. The results were expressed as means ± standard deviation.

RESULTS AND DISCUSSION

Sociodemographic profile of producers and consumers of local beers

The results of survey revealed that women aged between 20 and 40 represent the majority of the brewers (Table 1). They are predominantly catholic Christians, followed by Protestants and a few animists for all beers. Most of women are married (≥ 60%), followed by widowed, single or divorced. Their education level varies from primary to secondary school and illiterate as well (Table 1). In Moundou, Tchad, similar results were reported by Djitod (2002) on *Bil-bil* beer who found that the majority of producers are catholic Christians (87%), aged between 26 and 35 years. Also, they are married women (65%), divorced (15%), widows (13%) and single (7%). The producers of local beers in Maroua town were mainly women suggested that men are only the consumers that is consistent with the Northern tradition of Cameroon.

Results also showed that the youngest people aged between 20 and 40 years (75%) are mainly consume *Bil-Bil* beer (Table 1). Also, it found that *furdu* is more consumed by Protestants (57.7%) than others. However, *Bil-bil* (52%) and *Cochette* (50%) are mostly consumed by catholic Christian (Table 1). Then, other Christians such as Pentecostist, Adventist, Presbyterians and Muslims are consumed all types of beers. The consumers are predominantly married (≥ 50%) for all the beers than widowed, single and divorced. They have a level of education mostly between secondary and higher education and a few proportion of primary and illiterate (Table 1).

Furdu and *Cochette* beers are mostly consumed by people with a primary level of education. Pupils (32%) and students (27.5%) are the consumers of *Bil-Bil* beer. *Furdu* and *Cochette* are mostly consumed by the elder person aged between 40 and 60 years with a percentage of 63% and 45%, respectively, followed by the youngest people who aged between 20 and 39 years (Table 1). Also, according to our survey, low-income men and married people, unemployed and rich people are consumed the local beers in different streets of Maroua town every day from the morning (5 a.m.) to the evening (8 p.m.). The consumption of all these craft beers may be due to their traditional value, interested in tasting (Einfalt 2021), therapeutic value and lower cost than the commercial beers (modern beer). Fact, certain untypical flavors in craft beers even have the potential of being perceived by the consumer as having higher quality compared to commercial beer (Ascher 2012).

Different cereals used for production of local beers in Maroua town

The results of survey revealed that various cereals are used to produce local beers *(Bil-Bil, Furdu* and *Cochette)* in Maroua town. Cereals mostly used are maize, rice, fonio and sorghum. Rice and white sorghum were the main cereal for *Cochette* (40%) and for *Furdu* beer, it was white sorghum (85%) (Figure 3). While, *Bil-Bil* was the mixture or all of these different cereals. The mixture of red and yellow sorghum (46%) followed by red and white sorghum (38%), red sorghum, S35 and red-yellow sorghum and S_{35} were the most used by *Bil-Bil* producers, respectively (Figure 3). The present results are consistent with the results found by Charles et al. (2018), who reported that in the North Cameroon, red sorghum ("djagari") was the main cereal for the production of *Bil-Bil* beer. The results of the survey regarding producers show that *Bil-Bil* beer which produced with the mixture of red sorghum exhibited a better drinking though beer of red sorghum was absent in Maroua town, while, yellow and withe sorghum were the best cereal for production of *Bil-Bil* beer (Figure 3).

Therefore, many factors can affect the quality of these local beers such as quality of heat during preparation (33%), type and germination of grain (18%) and other factors (Figure 4). These are consistent with the results reported by Olaniran et al. (2017) who stated that the quality of the final product (beer) is influenced by several variables, such as the quality of the raw material, type of malting, the applied preparation method of the wort, type of hops, type and quality of yeast, fermentation time, maturation, and pasteurization and filtration, among others.

Table 1. Results of survey (%) of the local beers collected from consumers and producers from different street of Maroua town. Values are rated answer concerning the consumption or production of *Bil-Bil*, *Cochette* or *Furdu* beers.

Parameter		*Bil-Bil*		*Cochette*		*Furdu*	
		Consumers	**Producers**	**Consumers**	**Producers**	**Consumers**	**Producers**
School level	Primary	22.5	39	50	60	57.1	50
	Secondary	32	41	20	20	22.9	20
	Higher	27.5	0	12.5	0	10	0
	Illiterate	10	20	17.5	20	10	30
Matrimonial status	Married	50	63	60	75	69	50
	Single	40	14	10	15	11	20
	Widow	4	20	17.5	10	20	30
	Divorced	6	3	12.5	0	0	0
Religion	Animist	4	7	12.5	15	22.9	20
	Protestant	33	13	30	5	57.1	0
	Presbyterian	1.5	0	0	0	0	0
	Muslim	3	0	0	0	0	0
	Pentecost	6.5	0	0	0	0	0
	Catholic	52	80	50	80	20	80
	Adventist	0	0	7.5	0	22.9	0
Age	[0-20[	10	3	20	5	8.5	10
	[20-40[	75	77	35	50	28.5	60
	[40-60[	15	20	45	45	63	30

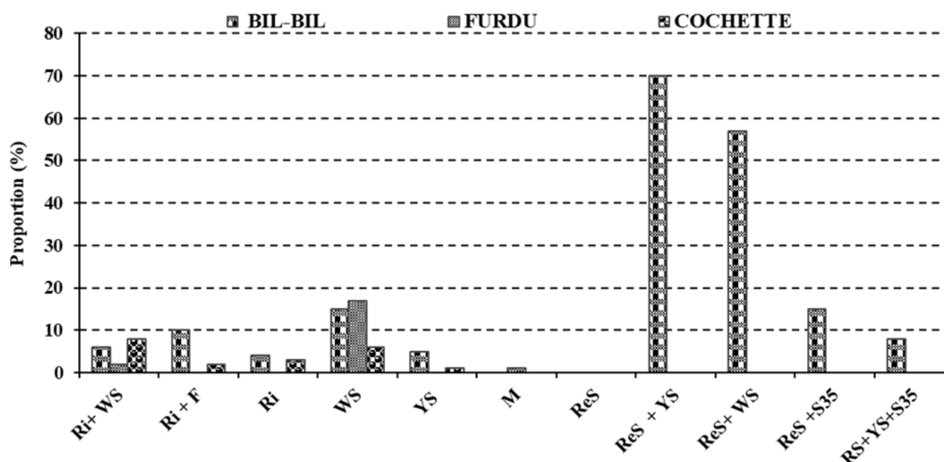

Figure 3. Proportion of the various combination of cereals used according to producers for craft brewery. Ri + WS = Rice + White *Sorghum*; Ri + F = Rice + *Fonio*; Ri = Rice, WS = White *Sorghum*, YS = Yellow *Sorghum*; M = Maize; RS = Red *Sorghum*; RS+YS = Red *Sorghum* + Yellow *Sorghum*; ReS + WS = Red *Sorghum* + White *Sorghum*; ReS+YS+S35 = Red *Sorghum* + Yellow *Sorghum* + S35.

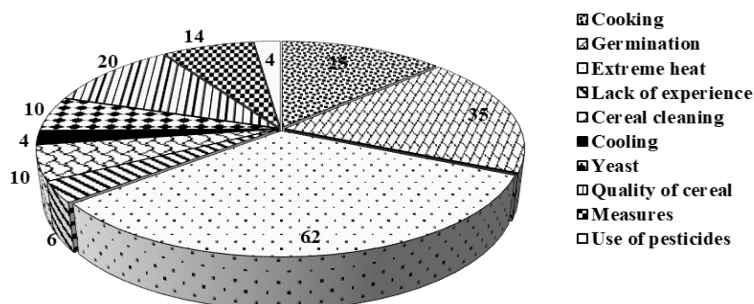

Figure 4. Factors influencing the craft brewery.

The influence of time and temperature on the volume of distillate

Figure 5.A-B showed the evolution of distillation of beers collected both morning and evening. The curve (Figure 5.A) showed many stages during the distillation. The curve showed that in the start time, all craft beers had a significant amount of alcohol. According to Anderson (2012) in a binary, tertiary or quaternary non-azeotropic mixture, the most volatile constituents are those whose boiling point is the lowest at constant pressure, which qualified as light at 1atm, and the least volatile constituent is the one with the highest boiling temperature at constant pressure qualified as heavy at 1atm. From the point of boiling temperature of methanol (64°C), ethanol (78°C), propan-2-ol (82°C), propana-1-ol (97°C) of water (100°C) and other products resulting from oxidation such as acetone (56°C). Samples CPiM, BPaM, CPvM, FHoM and BPvM for those collected in the morning (Figure 4.A) and BPvE, FHoE, CPvE, and BPaE for those of the evening (Figure 5.B) and specially at different time intervals lets us believe in their presence. Other samples FPvM (71°C) and BPiM (72°C) for those from the morning and FPvE (75°C) and BPiE (73°C) for those from the evening and their distillation time at a value of maximum boiling temperature strictly lower than that of ethanol is what also leads us to believe in an abundance of methanol, acetone and others.

These differences are due to the raw materials used, to the brewing methodology and the time and period of inoculation of the yeasts to obtain a perfect fermentation of alcohol. Fact, at temperature values strictly below and above the boiling point of ethanol there is no more ethanol to distillate in the local beer but many alcohols and other mixed products. Higher alcohols (2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol) derived from the catabolism of amino acids and sugars via the Ehrlich pathway are common in fermented and distilled beverages (Hazelwood et al. 2008; Nascimento et al. 2008). Gas chromatography (GC-FID) and GC-MS method for the detection and quantification of these different alcohols and other compounds in mixture has not been carried out in the present study, so we believe that there are many other compounds may be present. Recent works show many volatile compounds namely aldehydes, esters, alcohols and acids have been identified by using different chromatography method in cassava spirit and traditional sorghum beer (Attchelouwa et al. 2020; Coelho et al. 2020) which derived from the fermentative metabolism of yeast and can be further concentrated during the distillation.

Figure 5.C-D showed the evolution of the volume of distillate of various beers collected at different times (morning and evening). We noticed that the increase in volume at different times and temperatures varied in respect with the types of beers. All samples of beers from the morning to the evening had maximum boiling temperature with values greater than or equal to 78°C ($T \geq 78$°C) showed the highest value in terms of volume, 15.9 mL (BPvM), 15.8mL (CPiE) for low value and 20.85mL (CPiM), 21.3mL (BPvE) for high values. Almost ethanol content of sample beer was collected in different fractions, with distillation efficiency close to 100% (Coelho et al. 2020). On the other hand, we did not collect all the ethanol which was found in some samples, those whose boiling point was strictly below 78°C. We think it might take a longer time than we defined or at least there was not enough ethanol but other alcohols in those initial samples of beers.

Therefore, we obtained the smallest values in terms of volume with the value of 13.65 mL (BPiM) and 15.8 mL (FPvM) for the morning sample (Figure 4.C) and 15.8 mL (CPiE), 16.84 mL (BPiE) and 18.55 mL (FPvE) for those of the evening (Figure 4.D).

In general, the volume of distillate from the evening was significantly greater than those from the morning. This variation in volume between the samples collected in the morning and those from the evening may be due to the influence of the brewing technology, the raw material used (Humia et al. 2019) and the quality of yeast (Einfalt 2021) as well as time of maturation which have an effect on the fermentation of the different beers. This difference proves sufficiently that regardless of the temperature values greater than or equal to, less than or equal to the boiling temperature of ethanol $50 \leq Tb \leq 90$, we do not only collect ethanol and other alcohols mixed but also a significant amount of water. In short, at high temperature of around 100 °C, the distillate to be recovered consist of maximum water content and very low alcohol content.

Variation of some physicochemical characteristics in local beers

The physicochemical characteristics of *Bil-bil*, *Furdu* and *Cochette* are showed in Table 2. Statistical analysis showed a significant ($p<0.05$) difference among samples (Table 2). The results showed that TDS of all the investigated beers vary among them, however, *Furdu* beer has the highest TDS value (1688-1978 ppm), while the lowest was showed by *Cochette* (513-1409 ppm). The present results are in agreement with those reported by Coulibaly et al. (2020), who found in traditional sorghum beer (Tchapalo) in Ivory Coast a value of 15.60 °B which was variable than our values. This difference must be linked to the methodology using and the type of cereal to produce local beer. While, dry matter content of the beers varied between 4.24 to 6.98% *(Bil-Bil),* 2.83 to 6.96 % *(Furdu),* and 5.17 to 8.06 % *(Cochette).* Compared to the result obtained by Amane et al. (2005), the local beers investigated had a similar dry matter (7.87 %) of Sorghum *Tchapalo* produced in Ivory Coast. Similar results were also obtained by Chevauss-Agnes et al. (1976) who found that *Ambga* beer had 7.2 % of dry matter. These differences may be due to practices of production and the variety of cereals used in the processing of these beers.

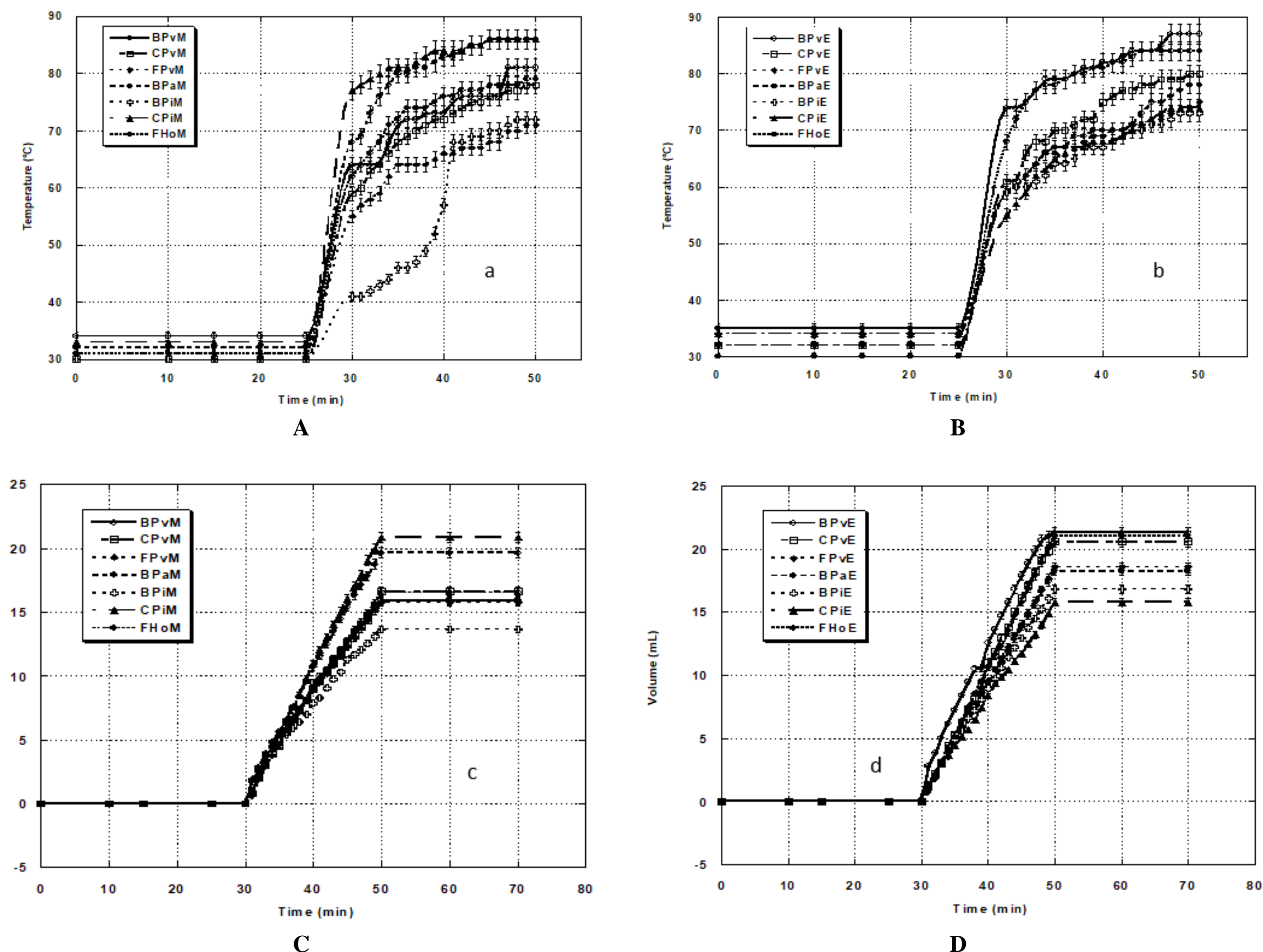

Figure 5. The distillation temperature and distillate volume according to the time of the local beers collected. A-B. Evolution of the distillation temperature according to the time of local beers collected (A. collected morning, B. collected evening). C-D. Increase in distillate volume according to the time of the local beers collected (C. collected morning, D. collected evening). Note: FHo: *Furdu* beer collected in *Ouro tchédé* street; FPv: Furdu beer collected in *Pont vert* street, CPv: *Cochette* beer collected in *Pont vert* street, CPi: *Cochette* beer collected in *Pitoaré* street, BPv: *Bil-Bil* beer collected in *Pont vert* street, BPa: *Bil-Bil* beer collected in *Palar* street, BPi: *Bil-Bil* beer collected in *Pitoaré* street. Those beers were collected in the evening (E).

The pH value of different beers varied among them as shown in Table 2. It varied from 3.53 to 3.37 *(Bil-Bil),* 3.84 to 4.15 for "Cochette" and 4.06 to 4.22 for furdu beer. Compared to the values obtained by Roger et al. (2013) in Amgba beer (pH 2.5), *Tchapalo* (pH 3.4) by Aka et al. (2008), *Dolo* (pH 3.5) by Abdoul-latif et al. (2013), *Pito* (pH 3.66) by Fadahunsi et al. (2013) and *Burukutu* (pH 3.94) by Eze et al. (2011) and sorghum safari (Tchapalo) (pH 3.5 to 4.5) by Coulibaly et al. (2020), all produced in Cameroon, Ivory Coast, Burkina-Faso, Nigeria and Ghana, respectively, our investigated beers had similar values. Also, it showed that all our local beers had similar pH value both in the morning and in the evening, which is in accordance with those reported by Maoura et al. (2006) in Chad, where the pH value of *Bil-Bil* had not changed during the alcoholic fermented process. However, the results revealed that all the sample beers had acidic pH range which can explained the fact that acidic medium is one of the main condition factors for the developing of yeast without any modification of pH (Maoura et al. 2006). The pH values of our local beers were within the desirable parameters, generally between 3.8 and 4.7, protecting the product against pathogens as reported by Suzuki et al. (2006). The difference in pH value may be attributed to the processing methods, quality of raw material and measure or quantification (Coulibaly et al. 2011). All values of pH of our different local beers produced in Maroua town is within the value of CODEX (2005), which reported that beer with a pH lower than 4.5 could be the good quality for the consumers.

The titratable acid of the local beers varied also significantly among samples ($p < 0.05$) (Table 2). *Bil-Bil* had the highest value (4.89-6.25 mg/mL), however *Frudu* had a value of 3.04-4.58 mg/mL, while *Cochette* had the lowest value (2.31-3.40 mg/mL). The values of titratable acid found in *Bil-Bil* beer are lower to the value (0.06mg/mL) reported by Coulibaly et al. (2020) in sorghum safari (Tchapalo). Similarly, these values are lower than those reported by Ourega et al. (2015), who found that plantain beer had a titratable acid content ranged

from 0.08 to 0.9.3 mg/mL suggesting that the raw material had influence on the beers.

For the alcohol content, results summarized in Table 2 revealed that the alcohol value varied significantly among beers ($P < 0.05$). The sample of *Furdu* beer had the contents varied between 5.40 and 6.92 %, while it folds from 1.08 to 5.10 % for *Cochette* and between 1.73 and 2.88 % for *Bil-Bil.* The *frudu* beer had alcoholic value higher than *Cochette* and *Bil-Bil* suggesting high flavour and aroma compounds (Einfalt 2021). However, the alcohol content in *Bil-Bil* beer was significantly higher than the value reported by Muyanja et al. (2010) on *Pito* (3.09 %), however lower than *Tchapalo* (5.22 %) processing with sorghum in Kenya, Nigeria and Ivory Coast, respectively. The rice and white millet used for the processing of *Furdu* (6.16%) and *Cochette* (3.09 %) had higher alcohol content than *Bil-Bil* (Table 2). These differences observed in terms of alcohol content are due to the types or quality of yeasts used to ferment. These are consistent with the results of Einfalt (2021) who found that the alcohol content varied significantly with the type of yeast used for beer fermentation. However, the alcohol levels obtained in our different beers are obviously within the range of the French legal classification of types of beers ranging from table beer to special beers brochures of brewers of France (Hencké 2000).

Reducing sugars content of *Furdu* varied between 11.85 and 19.05 mg/mL, while it fold from 2.20 to 9.46 mg/mL for *Cochette* and 9.92 to 19.78 mg/mL for *Bil-Bil* (Table 3). The present results showed that *Bil-Bil* beers had higher content of reducing sugars than *Tchapalo* (0.4 mg/mL) from Ivory Coast (Amane et al. 2005). This difference may be due to the reducing sugar content of sorghum used to process the local beers. Also, the contents of total carbohydrates varied among these beers (Table 3). The carbohydrates content in *Furdu* beer fold from 16.05 to 46.35 mg/mL, while in *Cochette* beer, it varied between 15.39 mg/mL and 42.53 mg/mL and for *Bil-Bil* the values varied from 12.12 to 168.53 mg/mL. It revealed that Bil-bil beer had lower and higher value of carbohydrates than that obtained in *Furdu* and *Cochette,* respectively. Cortacero-Ramírez et al. (2003) stated that beer should contain between 3.3 to 4.4 g/100 of carbohydrates. However, value is higher than that reported by Chevassus-Agnes et al. (1976) in *Ambga* and *Affouk* beers (19 mg/mL) consumed in the North region of Cameroon. The differences in carbohydrate values are linked to the variety of cereals and their technologies used for processing.

The values of soluble amino acids and total soluble proteins was not found varied significantly ($p > 0.05$) among the samples (Table 3). The soluble amino acids amount of the local beers were followed, *Furdu* had 3.40 to 7.37 mg/mL, *Cochette* contains 3.98 to 6.01 mg/mL, while *Bil-Bil* had 2.95 to 3.24 mg/mL. However, *Furdu* beer had the highest amino acid contents than *Cochette* and *Bil-Bil*. Considering the total proteins, local beers had high values ranged from 0.80 to 0.85 mg/mL which was higher than those obtained in *Kaffir* beer (0.05 mg/mL) (Busson et al. 1970), in *Amgba* (0.07 mg/mL) (Chevassus-Agnes et al. 1976) (Table 3). For Ferreira and Guido (2018), the contents of soluble amino acids determine the quality of beer suggesting the good quality of our sample beer analyzed.

The alcohol contents of distillate fractions from the morning and evening samples showed that the alcohol contents varied significantly ($p < 0.05$) among different samples of beers named FHoE, FPvE, CPiE, BPvE, and BPaE, however there is no significant difference among FHoM, FPvM, CPvM, CPiM, BiPvM and BPiE (Figure 6). The values of alcohol content revealed that the first fraction (F1) of the distillate had higher alcohol content than the second fraction (F2) (Figure 6).

Table 2. Total dissolved solids (TDS), pH, dry matter, titratable acid (TA) and alcohol contents of local beers (*Furdu*, *Cochette*, *Bil-Bil*) collected in the morning and evening from various streets in Maroua town.

Sample	TDS (ppm)	pH	Dry matter (g/100g)	T A (mg/mL)	Alcohol contents (g/100 mL)
FHoM	1771 ± 70^{b}	4.06 ± 0.03^{c}	6.96 ± 0.22^{b}	3.04 ± 0.19^{i}	5.38 ± 0.13^{b}
FHoE	1688 ± 23^{c}	4.06 ± 0.04^{c}	2.83 ± 0.08^{h}	3.80 ± 0.06^{g}	6.92 ± 0.28^{a}
FPvM	1939 ± 28^{a}	4.04 ± 0.02^{d}	6.96 ± 0.22^{b}	4.58 ± 0.32^{e}	5.47 ± 0.26^{b}
FPvE	1978 ± 20^{a}	4.22 ± 0.03^{a}	6.61 ± 0.32^{bc}	4.21 ± 0.06^{f}	5.40 ± 0.15^{b}
CPvM	1409 ± 26^{f}	4.15 ± 0.02^{b}	8.06 ± 0.08^{a}	2.63 ± 0.13^{j}	1.08 ± 0.23^{h}
CPvE	1310 ± 33^{g}	4.13 ± 0.06^{bc}	5.84 ± 0.08^{e}	3.40 ± 0.06^{h}	4.71 ± 0.21^{cd}
CPiM	625 ± 34^{h}	3.84 ± 0.03^{e}	6.09 ± 0.24^{d}	2.72 ± 0.13^{j}	4.31 ± 0.31^{d}
CPiE	513 ± 28^{i}	3.80 ± 0.03^{e}	5.17 ± 0.89^{f}	2.31 ± 0.06^{k}	5.10 ± 0.25^{c}
BPiM	1442 ± 18^{e}	3.46 ± 0.02^{g}	6.98 ± 0.37^{b}	5.07 ± 0.26^{d}	2.55 ± 0.25^{f}
BPiE	1434 ± 11^{ef}	3.45 ± 0.05^{g}	5.42 ± 0.44^{ef}	5.84 ± 0.32^{bc}	2.90 ± 0.23^{e}
BPaM	1495 ± 24^{d}	3.44 ± 0.02^{g}	4.88 ± 0.10^{f}	4.89 ± 0.26^{de}	1.73 ± 0.13^{g}
BPaE	1647 ± 29^{c}	3.53 ± 0.03^{f}	6.31 ± 0.07^{c}	5.66 ± 0.06^{c}	1.82 ± 0.26^{g}
BPiM	1483 ± 34^{de}	3.42 ± 0.04^{gh}	4.24 ± 0.09^{g}	6.25 ± 0.13^{a}	1.92 ± 0.13^{g}
BPiE	1441 ± 11^{ef}	3.37 ± 0.02^{h}	5.95 ± 0.38^{de}	5.93 ± 0.06^{b}	2.88 ± 0.21^{ef}

Values are means ± standard deviation of tree replicates (n= 3). In the same column, values followed by different superscript letters are significantly different ($p < 0.05$). Note: FHo: *Furdu* beer collected in *Ouro tchédé* street; FPv: *Furdu* beer collected in *Pont vert* street; CPv: *Cochette* beer collected in *Pont vert* street; CPi: *Cochette* beer collected in *Pitoaré* street; BPv: *Bil-Bil* beer collected in *Pont vert* street; BPa: *Bil-Bil* beer collected in *Palar* street; BPi: *Bil-Bil* beer collected in *Pitoaré* street. Those beers were collected in the morning (M) or evening (E).

Table 3: Reducing sugars, total amino acids, total carbohydrates, and total proteins of local beers (*Furdu*, *Cochette*, *Bil-Bil*) collected in the morning and evening from various streets in Maroua town. The values are expressed in mg/mL of sample.

Sample	Reducing sugars	Carbohydrates	Total protein	Amino acids
FHoM	16.35 ± 0.45^{d}	16.05 ± 0.27^{h}	0.80 ± 0.05^{b}	4.29 ± 0.19^{c}
FHoE	17.33 ± 0.63^{c}	37.75 ± 0.99^{d}	0.83 ± 0.03^{ab}	3.40 ± 0.13^{e}
FPvM	11.85 ± 1.02^{f}	46.35 ± 1.14^{b}	0.84 ± 0.02^{a}	6.00 ± 0.16^{a}
FPvE	19.05 ± 0.63^{a}	44.71 ± 1.50^{bc}	0.83 ± 0.02^{ab}	7.37 ± 0.15^{a}
CPvM	8.30 ± 0.54^{h}	35.97 ± 1.17^{d}	0.83 ± 0.06^{ab}	5.83 ± 0.17^{b}
CPvE	9.46 ± 0.37^{gh}	42.53 ± 0.63^{c}	0.84 ± 0.05^{a}	3.98 ± 0.15^{d}
CPiM	2.20 ± 0.58^{j}	28.34 ± 0.89^{e}	0.83 ± 0.04^{ab}	6.01 ± 0.07^{b}
CPiE	5.56 ± 0.22^{i}	15.39 ± 0.55^{h}	0.83 ± 0.04^{ab}	4.44 ± 0.09^{c}
BPvM	13.06 ± 0.65^{e}	19.00 ± 0.80^{g}	0.81 ± 0.02^{b}	3.15 ± 0.17^{f}
BPvE	9.92 ± 0.41^{g}	12.12 ± 0.69^{i}	0.81 ± 0.02^{b}	3.03 ± 0.13^{gf}
BPaM	17.51 ± 0.84^{bc}	18.81 ± 0.43^{g}	0.83 ± 0.04^{ab}	3.02 ± 0.16^{gf}
BPaE	16.32 ± 0.71^{d}	168.53 ± 1.52^{a}	0.83± 0.02^{ab}	3.01 ± 0.04^{g}
BPiM	18.02 ± 0.48^{b}	24.49 ± 0.79^{f}	0.85 ± 0.03^{a}	2.95 ± 0.08^{g}
BPiE	19.78 ± 0.58^{a}	27.80 ± 1.04^{e}	0.80 ± 0.02^{b}	3.24 ± 0.10^{ef}

Values are means ± standard deviation of tree replicates (n= 3). In the same column, values followed by different superscript letters are significantly different ($p < 0.05$). Note: FHo: *Furdu* beer collected in *Ouro tchédé* street; FPv: *Furdu* beer collected in *Pont vert* street; CPv: *Cochette* beer collected in *Pont vert* street; CPi: *Cochette* beer collected in *Pitoaré* street; BPv: *Bil-Bil* beer collected in *Pont vert* street; BPa: *Bil-Bil* beer collected in *Palar* street; BPi: *Bil-Bil* beer collected in *Pitoaré* street. Those beers were collected in the morning (M) or evening (E).

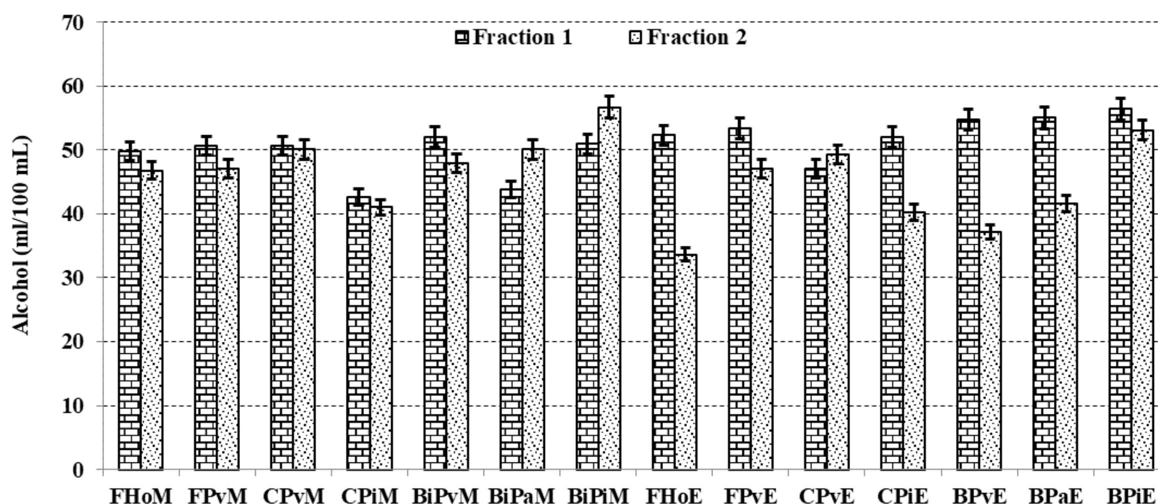

Figure 5. Alcohol contents (mL/100 mL) of distillate obtained from local beers (*Furdu*, *Cochette*, *Bil-Bil*) collected in the morning and evening from serval streets in Maroua town, Cameroon. Note: FHo: *Furdu* beer collected in *Ouro tchédé* street; FPv: *Furdu* beer collected in *Pont vert* street; CPv: *Cochette* beer collected in *Pont vert* street; CPi: *Cochette* beer collected in *Pitoaré* street; BPv: *Bil-Bil* beer collected in *Pont vert* street; BPa: *Bil-Bil* beer collected in *Palar* street; BPi: *Bil-Bil* beer collected in *Pitoaré* street. Those beers were collected in the morning (M) or evening (E).

This result may be due to the fact that at the start of the distillation, a strong alcohol type has been collected with very strong odor with a sour taste and a characteristic aroma which relatively increased the alcohol content (Aka et al. 2008). On the other hand, at the end of the distillation, we observed an increase in temperature and consequently an alteration of the distillate and other volatile compounds which decreased the alcohol content by water interference. In general, ethanol was the main constituent of distillate, however this does not characterize the quality of the distillates. As indicated by Huot and Roy (1999), other flavors from many organic substances such as esters, aldehydes and other alcoholic compounds must also interfere in the alcoholic degree of the distillates. These compounds may influence the quantity and quality of distilled ethanol. An ethyl acetate which had also been found in cassava spirits indicated that the distilled beverages such as whiskey, rum and Cachaça affected the quality of them (Coelho et al. 2020).

At less significant values, samples of distillates collected in the evening are richer in alcohol than those obtained in the morning, suggesting that this fraction was rich in flavor compounds. Fact, the principal high alcohol in alcoholic beverages include 1-propanol, 2-methyl-1-

propanol (isobutanol), 3-methyl-1-butanol (isoamylol) and 2-phenylethanol (Zhou et al. 2020). Likewise, some sample saw their second fraction more concentrated than the first one (Figure 6). This difference could be due specially to the yeast inoculation time (Einfalt 2021) and the fermentation time because early in the morning some local beers had not reached their fermentation or maturation threshold and can affect the quality of the distillate. Fact, the fermentation process develops with an increase in the alcohol level (Périsse et al. 1959).

In conclusion, the aim of this present study was to investigate the physicochemical characteristics of various local beers produced and consumed in Maroua town as well as the types of cereals used to produce these craft beers. Various cereals (red sorghum, white sorghum, maize, yellow millet, S35, rice and fonio, etc.) are the mainly used to process different local beers. The volume of distillate increased by time and depend on one beers to another. The sample of BPiM and CPiM had the lowest and the highest temperature values during distillation, respectively. *Furdu* beer had the highest TDS value (1688-1978 ppm), pH value (4.06-4.22) and high level of alcohol (5.40-6.92 %). However, *Cochette* had the highest level of dry matter (5.17-8.06 %). *Bil-Bil* had the highest titratable acid value (4.89 mg/mL-6.25 mg/mL). The distillate of the local beers had high alcohol levels and varied from beer to another and one fraction to another, with the values varied between 30 and 58.03%. These results revealed that various cereals would be suitable as raw materials for the production of quality beers such as white beers, beers, and spirits and provide a basis to foster the production of value-added products from these cereals. This study shows that the local beers are rich in nutriment and could offer a greater source of benefit to consumers.

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Comparative determination of total antioxidant effects of ethanol extract of *Phyllanthus amarus* leaves

OJOCHENEMI EJEH YAKUBU, MICHAEL SUNDAY ABU♥, JOHN AKIGHIR, JOB ITANYI ONUCHE, ALI ARABI
Department of Biochemistry, Faculty of Pure and Applied Sciences, Federal University Wukari. PMB 1020 Katsina-Ala Road, Wukari, Taraba State, Nigeria. Tel. +234-803-0000000, ♥email: abulex77@yahoo.com

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Abstract. *Yakubu OE, Abu MS, Akighir J, Onuche JI, Arabi A. 2021. Comparative determination of total antioxidant effects of ethanol extract of* Phyllanthus amarus *leaves. Asian J Nat Prod Biochem 19: 81-85.* Some medicinal and therapeutic potency have been reported of *Phyllanthus* in traditional settings. Hence, the current study evaluated the total antioxidant potential, total phenolics and total flavonoids of fractions of ethanol extract of *Phyllanthus amarus* Schumach. & Thonn. leaves obtained using column chromatography. Extraction was done using absolute ethanol while elution was performed using different organic solvents of varying polarities, from n-hexane, chloroform, ethyl acetate, ethanol, methanol and finally water. The results obtained from the study revealed that fraction 10b (83 mg/mL), has the highest concentration of Total antioxidant capacity (TAC), the next being 1a, 5a, 11b (48 mg/mL). The reduction in the TAC of the fractions was in the order; 3b>7a>6a>5b>4a> with F7b (13mg/mL) has the lowest TAC. The result for Total phenolic content (TPC) shows that fraction 10b (430 mg/mL), has the highest concentration of TPC, just like in antioxidant activity, followed by F4b (428 mg/mL). Fraction 2a (9 mg/mL) has the lowest concentration of TPC. Total flavonoid content (TFC) showed that fraction 3b (201 mg/mL) has the most concentration of total flavonoids, followed by 4b (120 mg/mL). Meanwhile, fractions 1a, 2b and 9a have the same TFC. While fraction 2a showed the lowest concentration (25 mg/mL) of flavonoids. The plant extract fractions were found to contain more phenolics and flavonoids, which may have contributed to the observed antioxidant activity.

Keywords: Antioxidant, ethanol extract, flavonoids, phenolic, *Phyllanthus amarus*

INTRODUCTION

Phyllanthus amarus Schumach. & Thonn. is of the Euphorbiaceae family while the genus is *Phyllanthus*. The species can be readily found in tropical and subtropical countries of the world (Trease and Evans 2001). It is known in Nigeria, among Yoruba as “eyin olobe”, Hausa as “geerontsuntsaayee” and Igbo as “Ite knwonwa nazu” and in English as leaf flower or stone breaker. The plant is usually prepared as herbal mixture and used by Nigerian for health purpose and is considered to have great economic importance (Umoh et al. 2013). Previously, the whole plant extract has been used for treatment of urinary disorders, liver disease, insecticide, dyspepsia, anorexia, constipation and dysentery (Samy et al. 2008; Ankutse et al. 2021), while many female anomalies like leucorrhoea, menorrhagia, mammary abscess have been managed by this plant exudate (Samuel and Andrews 2010). Again, fresh leaf paste prepared from *P. amarus* has the potential to alleviate white spots on skin, manage diabetes, and reduce the incidence of jaundice (Ignacimuthu et al. 2008). Similarly, malaria has been successfully managed by *P. amarus* whole plant extract (Kuppusamy and Murugan 2010).

Methanolic extract of *P. amarus* was found to have antioxidant effect that inhibited lipid oxidation and masked up reactive oxygen species in-vitro (Meena et al. 2018) while ethanolic extract of *P. amarus* hairy roots showed antiproliferative activity against apoptosis initiated by increased intracellular reactive oxygen species (Abhyankar et al. 2010). The effect of nor-securinine, an alkaloid extracted from *P. amarus* was examined against spore growth of some fungi as well as powdery pea mildew (*Erysiphe pisi*) and was found to be potent (Meena et al. 2018). *Phyllanthus amarus* ethanol, aqueous and hexane extracts showed restriction of LPS-induced production of NO and PGE2, and also decreased the LPS-induced secretion of Tumor necrosis (Kiemer et al. 2003). The combination of *P. amarus* and *Andrographis paniculata* plant extracts demonstrated active snake anti-venom potential that could be useful against snakebite (Meena et al. 2018). Similarly, Sornakumar et al. (2014) reported that about 0.24 mg of di-herbal plant extracts comprising *P. amarus* effectively neutralized the cobra venom induced toxicity.

Certainly, *P. amarus* has been reported to have some medicinal and therapeutic potency in traditional settings. These medicinal properties are mostly implicated with the presence of phytochemicals which are believed to exert some specific pharmacological effects on the body. Hence, the current study evaluated the total antioxidant capacity, total phenolics and total flavonoids of GC-MS partially fractionated ethanol extract of *P. amarus* leaves.

MATERIALS AND METHODS

Plant material

Fresh healthy looking leaves of *P. amarus* were collected within the premises of Federal University Wukari, Taraba State, Nigeria. *Phyllanthus amarus* was identified and authenticated by Dr. Yakubu J.O.E, a Pharmacologist, in the Department of Biochemistry, Faculty of Pure and Applied Science Federal University Wukari, Taraba State, Nigeria. The leaves were air dried in the laboratory at 25ºC for a period of one week and pulverized manually using laboratory mortar and pestle into smooth particle and stored in an air-tight container for further use.

Solvent extraction

About 500 g of the pulverized plant leaves were soaked in 1.3 L of 97% ethanol for 48 hours at 25ºC with periodic shaking to ensure maximum extract yield, according to Yakubu et al. (2014). The resulting crude extract was filtered using clean white sieving mesh and whatman number 1 filter paper. The filtrate was evaporated to dryness with a rotary evaporator at low temperature under reduced pressure to obtain green crude extract.

Fractionation of ethanol extract by column chromatography

The ethanolic green crude extract was subjected to column chromatography to separate the extract into its component fractions. Silica gel was used as stationary phase while different solvents of increasing polarity were used as the mobile phase. According to Yakubu et al. (2014), the bottom part of the glass column was packed with glass wool with the help of glass rod. Exactly 235 g of silica gel of mesh size 60-200 was dissolved in 225 mL absolute n-Hexane to make the slurry (activation of silica gel). The chromatographic column (30 mm diameter by 40 mm height) was packed with silica gel and the solvent was allowed to flow into a conical flask below freely. At the end of the packing process, all taps were locked and the setup was allowed to stand for 24 hours, and the clear solvent at the top of the silica gel was allowed to drain down the meniscus.

Elution

The Yakubu et al. (2014) method was adopted for the elution. Two grams of the ethanol extract of *P. amarus* leaves was dissolved in 15 % absolute ethanol and the solution was applied into the chromatography column (30 mm in diameter and 400 mm in height) followed by elution of the extract using n-hexane, chloroform, ethyl acetate, ethanol, methanol, and finally distilled water. The following ratios (v/v) in mL of solvent combinations were frequently used in the elution process: n-hexane: chloroform 100:0, 50:50; chloroform: ethyl acetate 100:0, 50:50; ethyl acetate: ethanol 100:0, 50:50; Ethanol: methanol 100:0 50:50; Methanol: distilled water 100:0 50:50; distilled water:100. A measured volume (200 mL) of each solvent combination was poured into the column each time using separator funnel. The eluted fractions were collected in aliquots of 100 mL in conical flask.

Determination of total antioxidant capacity (TAC) (Singleton et al. 1999)

The absorbance was measured in triplicate for each fraction. Total antioxidant capacity (TAC) was calculated as mg/mL of trolox equivalent (TE) using the regression equation from the calibration curve. Exactly 0.05g of the extract was dissolved in 250 mL of methanol in order to prepare 0.2 mm. One mL of methanol extract was added to 2 mL of DPPH in each test tube followed by the addition of 100 µL of fraction to each test tube. The mixture was shaken vigorously and left in dark at room temperature for 30minuts. The absorbance of the solution was measured immediately at 517 nm in a UV-Visible spectrophotometer. The experiment was performed in duplicate.

Determination of total flavonoids content (TFC) (Chang et al. 2002)

Quercetin standard was used for derivation of the calibration curve. The total flavonoids were express as mg/mL quercetin equivalent (QE). Solution of 10% aluminium chloride was prepared by dissolving 10 g of aluminium chloride ($ALCL_3$) in 100 mL ethanol. Exactly 100 µL of diluted sample was added to test tube containing 1.5 mL methanol followed by the addition of 100 µL of aluminium chloride ($ALCL_3$) and 100 µL potassium acetate solutions. The mixture was incubated at room temperature for 30minuts. The absorbance of reaction mixture was measured at 765 nm wavelength using UV-Visible spectrophotometer using distilled water as the blank.

Determination of total phenolic content (TPC) (Yakuku et al. 2014)

Folin-Ciocalteu reagent was diluted (1:10 using distilled water). A volume of 100 µL of crude sample was added into each test tube and 2.5 mL of Folin-Ciocalteu regent was added into each test tube followed by 2 mL of Na_2CO_3 solution. The solution was incubated for 15 minutes at 45ºC. The absorbance was measured at 415 nm in UV-Visible spectrophotometer using distilled water as the blank.

RESULTS AND DISCUSSION

Total antioxidant capacity (TAC)

Figure 1 shows total antioxidant capacity (TAC) of fractions obtained from ethanolic extract of *P. amarus* leaves. Fraction 10b (83 mg/mL), has the highest concentration of total antioxidant capacity, followed by F1a, F5a, and11b that all have similar value of TAC (48 mg/mL). Other fractions, F4b, F9b, F10a are also of the same value (44 mg/mL) while fractions, 1b, 2a, 2b, 3a, 6a, 8b, and 9a (23 mg/mL) possess the same TAC. The decrease observed in the TAC of the fractions is in the order of F3b>F7a>F6a>F5b>F4a>, with F7b (13 mg/mL) showing the lowest total antioxidant capacity.

Total phenolic content (TPC)

The result for total phenolic content of ethanol extract of *P. amarus* leaves shows that fraction 10b (430 mg/mL), has the highest concentration of total phenolic content as it was in the case of antioxidant activity. This was followed by F4b (428 mg/mL), while fraction 2a (9 mg/mL) has the lowest concentration of TPC as shown in Figure 2.

Total flavonoid content (TFC)

The Total flavonoids content (TFC) of ethanol extract of *P. amarus* leaves revealed that fraction 3b (201 mg/mL) possess the highest concentration of total flavonoids content as depicted in Figure 3, followed by 4b (120 mg/mL). Fractions 1a, 2b, 9a have the same TFC. The decrease observed in the TFC of the fractions is in the order of 8b>5a>5b>7a>11a>7b. Meanwhile, F2a has the lowest concentration (25 mg/mL).

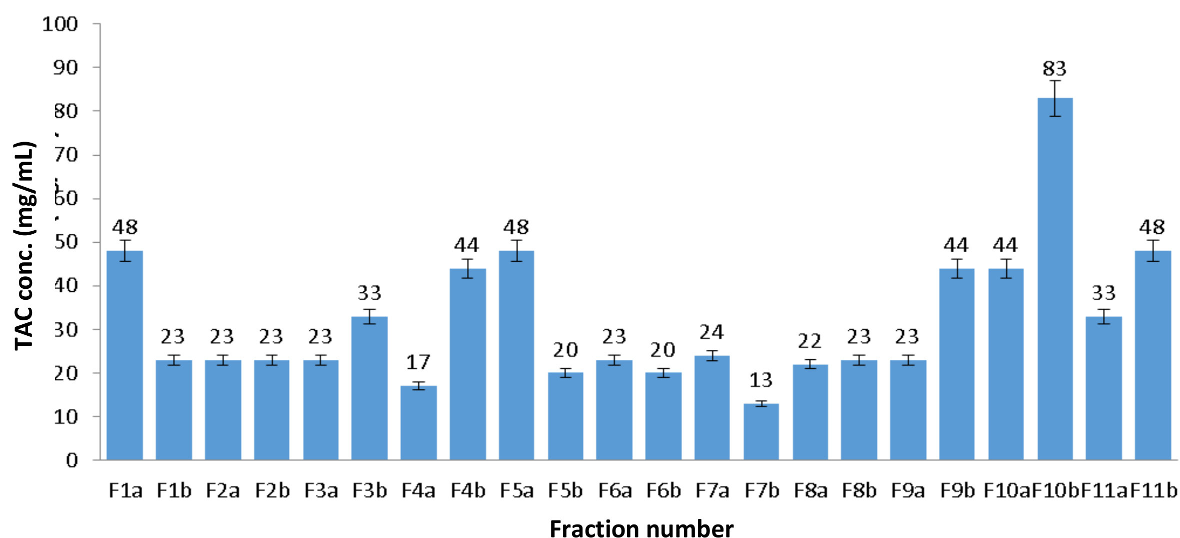

Figure 1. Total antioxidant capacity (TAC). F1: n-Hexane: (100:0), F2: n-Hexane/chloroform: (50:50), F3: Chloroform: (100:0), F4: Chloroform/ethyl acetate (50:50), F5: ethyl acetate: (100:0), F6: Ethyl acetate/ethanol (50:50), F7: Ethanol (100:0), F8: Ethanol/ Methanol: (50:50), F9: Methanol: (100:0), F10: Methanol/water (50:50), F11: Distilled water: (100:0)

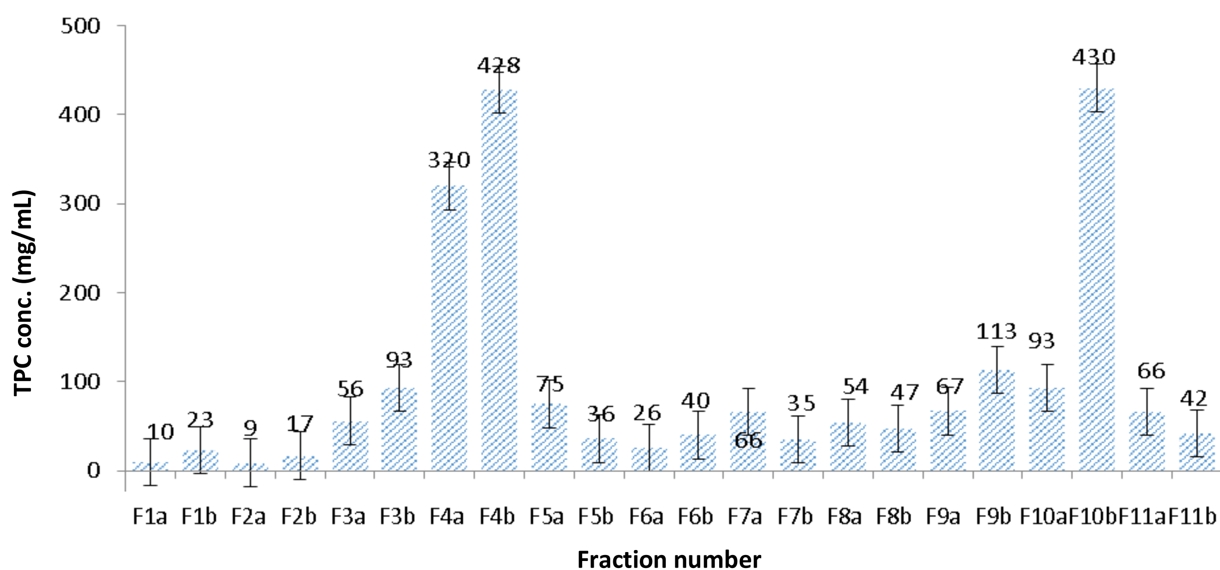

Figure 2. Total phenolic content (TPC). F1: n-Hexane: (100:0), F2: n-Hexane/chloroform: (50:50), F3: Chloroform: (100:0), F4: Chloroform/ethyl acetate (50:50), F5: Ethyl acetate: (100:0), F6=Ethyl acetate/ethanol (50:50), F7=Ethanol (100:0), F 8: Ethanol/ Methanol: (50:50), F9: Methanol: (100:0), F10: Methanol/water (50:50), F11: Distilled water: (100:0)

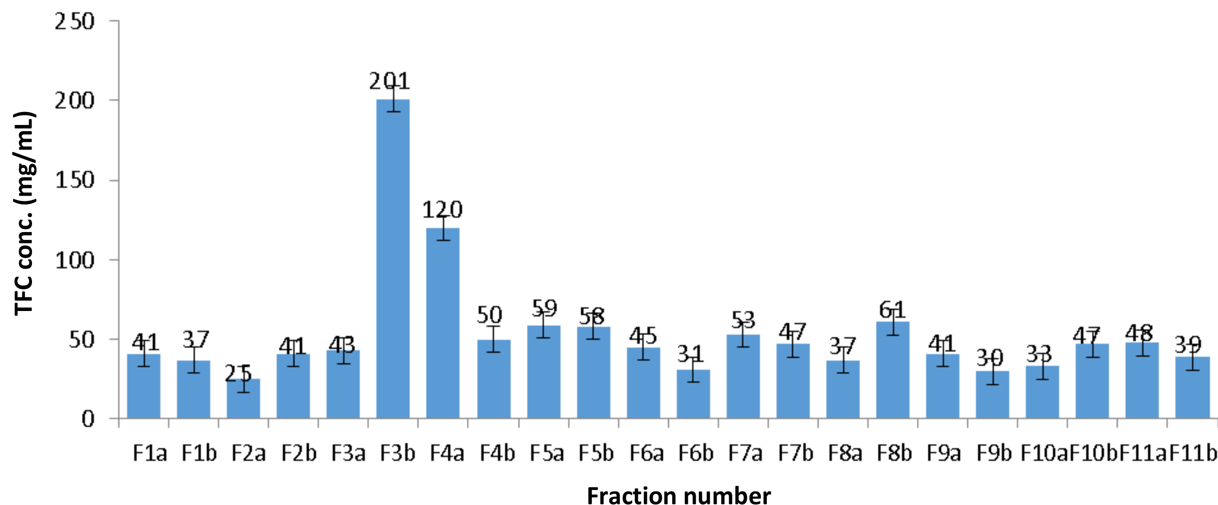

Figure 3. Total flavonoid content (TFC). F1: n-Hexane: (100:0), F2: n-Hexane/chloroform: (50:50), F3: Chloroform: (100:0), F4: Chloroform/ethyl acetate (50:50), F5: Ethyl acetate: (100:0), F6: Ethyl acetate/ethanol (50:50), F7: Ethanol (100:0), F8: Ethanol/ Methanol: (50:50), F9: Methanol: (100:0), F10: Methanol/water (50:50), F11: Distilled water: (100:0)

Discussion

Phyllanthus amarus extract has attracted several researches over the decades because of its potent pharmaceutical uses. Every country has their own traditional use of *P. amarus* but the way of curing disease is almost common everywhere (Meena et al. 2018). Antioxidant compounds usually deactivate free radicals and inhibit the process of lipid peroxidation, which is one of the major reasons for the deterioration of food and biological cells (Halliwell 2010).

In this study, a considerable high level of antioxidant activity was observed in the ethanol extract of *P. amarus* leaves. The TAC revealed that fractions 10b (84 mg/mL), 1a, 5a (48 mg/mL), possessed higher antioxidant capacity which implies that, the fractions have higher antioxidant values when compared with the other fractions. This observation was substantiated with the positive correlation that was seen between the TAC and the concentration of phenolic compounds present in the fractions; since it has been demonstrated by several studies that, the higher the phenolic content the higher the TAC. The result also indicated that, the solvent combination methanol-water (50:50) is the most efficient solvent combination for elution of antioxidant activity of ethanolic extract of *P. amarus* leaves as reported by Yakubu et al. (2018). However, the low antioxidant capacity observed in fractions 4a (17 mg/mL) and 7b (13 mg/mL), could be attributed to the fact that antioxidant activity depends on the type, polarity of the extracting solvent and the purity of active compounds (Demiray et al. 2009).

Plants have diverse groups of phenolic compounds, which include simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids, which have radical scavenging power (Olamide et al. 2017). The result obtained for the TPC shows that fraction 10b (430 mg/mL), has the highest concentration of total phenolic content**.** Yakubu et al. (2019), stated that, phenolic compounds are the most effective and abundant natural antioxidants in plants. They are very important plant constituents because their hydroxyl groups confer scavenging ability (Motawi et al. 2011). Thus, it can be inferred with certainty that, these phenolics are responsible for the marked antioxidant activity of these fractions which is compatible with several studies reported on the relationships between phenolic content and antioxidant activity (Yakubu et al. 2018).

In conclusion, the different methods employed in the study revealed that, the ethanolic extract of *P. amarus* leaves are rich in phenolic and flavonoids compounds and demonstrated good antioxidant activity, and can be useful in masking up free radicals as assayed through various in vitro models in this study. Thus, preventing free-radical related disorders respectively. There appears to be no correlation between antioxidant activity and flavonoid content, which implies that the plant extract contains several phytochemicals other than flavonoids which may contribute to the antioxidant capability. Hence, supporting the use of this plant as good source of natural antioxidant should be encouraged.

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