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Full Length Research Paper

Systematic review: Medicinal use and scientific elucidation of the *Piper* genus for the treatment of symptoms and inflammatory diseases

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The aim of this study was to conduct a systematic review that reported the medicinal use of the genus *Piper,* and pharmacological elucidations for the treatment of symptoms and processes of inflammation and inflammatory diseases. The systematic review was prepared in accordance with PRISMA guidelines. The databases used for this research were the Web of *Science* and Scopus, where associations with the terms were applied in both databases: "*Piper*" *and* "*Ethnobotanical*" and "*Piper*" *and* "*Anti-inflammatory effect*". Initially, the research identified 153 articles, of which 24 articles were selected for final analysis following the inclusion criteria. The results indicate that the medicinal use of species of the *Piper* genus entails mainly using of leaves, roots, and fruits, and decoct, maceration and powder for the treatment of inflammatory and respiratory diseases such as asthma and bronchitis. Regarding the evaluation of the anti-inflammatory effect of the genus, only seven species presented studies with their scientifically proven anti-inflammatory effect. Biases in the methodology applied in the studies were observed. In this systematic review, it was noticed that both studies addressed have gaps that can cause damage to future research and that pharmacological studies for inflammation with the use of the species themselves are still limited, with a more job for the isolated compounds of these plants.

Key words: *Piper, medicinal,* ethnobotanic, anti-inflammatory effect.

INTRODUCTION

Piper genus is the most representative of Piperaceae family, composed of about 1,000 species (Durant-Archibold et al., 2018), and has wide distribution on temperate regions from both hemispheres (tropical and sub-tropical). It is used for cooking, and has aromatic, ornamental and medicinal purposes (Reigada et al., 2007; Santos et al., 2012). In Brazil, it can be found from North to South, where 292 species occur, of which 44 are

variety and 184 are endemic (Flora do Brasil, 2018).

Concerning the botanical aspects, these genus specimens appear as sub-shrubs, shrubs or trees, measuring from 1 to 5 meters; they are able to reach up 10 meters in height. The stems are usually lignified, knotty and have many branches. Leaves have a long petiole and alternate with the stem. The limbo is simple, having its entire margin, with different shapes and sizes. The flowers are sessile, arranged in spikes opposite to leaves, varying in length and thickness, upright, sub-curved or curved; they are accompanied by thin or thick peduncles, of 2 to 5 stamens and 3 to 4 stigmas, filiform, curved, styled or sessile. The ovary is ovoid or sub-ovoid. Fruits are globular drupe with little thickened pericarp (Reitz, 2003; Guimarães and Giordano, 2004).

Ethnobotanical studies in the literature exhibit the genus *Piper* related to medicinal use in tea forms (decoction), infusion and aromatic baths (Blumenthal, 1998; Santhakumari et al., 2003; Wirotesangthong et al., 2008). It is mostly species used in traditional medicine for treating gastrointestinal diseases, hypertension, anti-hemorrhagic, diuretic, pain and inflammation (Gupta et al., 2015; Reigada et al., 2007; Roersch, 2010).

Species of this genus produce compounds with diverse biological and pharmacological properties, such as anxiolytic, analgesic, anti-inflammatory, vasodilatory, cytotoxic, immunomodulatory, antimicrobial, antifungal and promising antitumor activities (Bezerra et al., 2008; Rodrigues et al., 2009; Moraes et al., 2011; Raj et al., 2011). In this sense, phytochemistry contributes to verifying *Piper* genus biological activities cited in the ethnobotanical researches (Parra et al., 2011).

Phytochemical research brings about numerous scientific studies in many parts of the world, which has led to isolation of several bioactive compounds such as kavalactones (Xuan et al., 2008; Whitton et al., 2003), aristolactams (Cardoso Júnior and Chaves, 2003; Chaves et al., 2006), phenylpropanoids (Chaves and Santos, 2002), lignoids (Chen et al., 2007; Bodiwala et al., 2007), chromones (Morandim et al., 2005), terpenes (Baldoqui et al., 2009; Péres et al., 2009), steroids (Parmar et al., 1997), prenylated benzoic acids (Lago et al., 2009; Chaves et al., 2010) and amides (Araújo-Júnior et al., 1997; Chaves et al., 2003; Srinivasan, 2007; Cotinguiba et al., 2009). These characterize the metabolites of this genus. The first isolated amide of the Piper genus was piperine (Lee et al., 1984).

The main biological activities attributed to isolated substances are antifungal, insecticidal, bactericidal, antitumor, trypanocidal, antiparasitic, antimicrobial, antiprotozoal, anti-inflammatory, antinociceptive and antioxidant (Pohlit et al., 2004; Nakamura et al., 2006; Deshwal, 2013; Zakaria et al., 2010; Agbor et al., 2012).

A striking feature of the genus is presence of rich oil content in its structures, especially in leaves and fruits (Albiero et al., 2005). It has essential oils with chemical constituents such as monoterpenes, sesquiterpenes, arylpropanoids, aldehydes, ketones, and long-chain alcohols. This shows its commercial and industrial potential, as well as use in traditional medicine (Correa et al., 2011)

Some studies of scientific reviews on species and isolates from *Piper* genus have already been published (Roersch, 2010; Gutierrez et al., 2013; Monzote et al., 2017, Durant-Archibold et al., 2018); however, there is no systematic review of the genus *Piper* focusing on inflammatory processes. Hence, this review aims to report the medicinal use of *Piper* genus, including pharmacological elucidation for the treatment of symptoms of inflammation and inflammatory diseases.

MATERIALS AND METHODS

Research strategy

The systematic review was performed according to PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). It consists of a 27 items checklist and a flow diagram that instructs articles search and selection in four steps - identification, selection, eligibility and inclusion - for authors to contribute to the quality and reliability of the review in question (Moher et al., 2009).

The study was guided by two questions. Ffirst: "Which species of *Piper* genus are used empirically by population for symptoms of inflammation and inflammatory diseases treatment"? The second: "Which species of the genus *Piper* have scientifically elucidated anti-inflammatory effects?"

The articles mentioned in the review were publications available from January 2003 to July 2017. For this research, the Web of Science and Scopus databases were used, where the following association of terms was applied in both bases: "*Piper*" and "Ethnobotanical", "*Piper*" and Anti-inflammatory effect. The constancy of the terms aims to guarantee greater trustworthiness in search application forms and later comparison of the publication date.

The inclusion and exclusion criteria were applied to both consulted databases. Inclusion criteria: articles published in full text, in English language and that portray the subject by means of association of the above-mentioned terms. Exclusion criteria: duplicate articles, comparative, inconclusive, or dubious studies. After the use of research criteria, certification of selected productions was carried out, using a meta-synthesis type qualitative approach.

Studies selection

Two independent researchers (C.N.F.L. and L.F.L) made studies research and selection. The choice of articles was made by reading title and abstract, with the application of an evaluation formulary with eligibility criteria. Later, publications were evaluated by complete reading. This step is indispensable to ratify the inclusion criteria. Any divergences were agreed on between the two researchers.

Methodological quality analysis/studies' bias risk

The studies were evaluated using SYRCLE's RoB (Hooijmans et al., 2014) tool, for pre-clinical *in vivo* studies, with non-human animals. Related to ethnobotanical and pre-clinical studies *in vitro* it
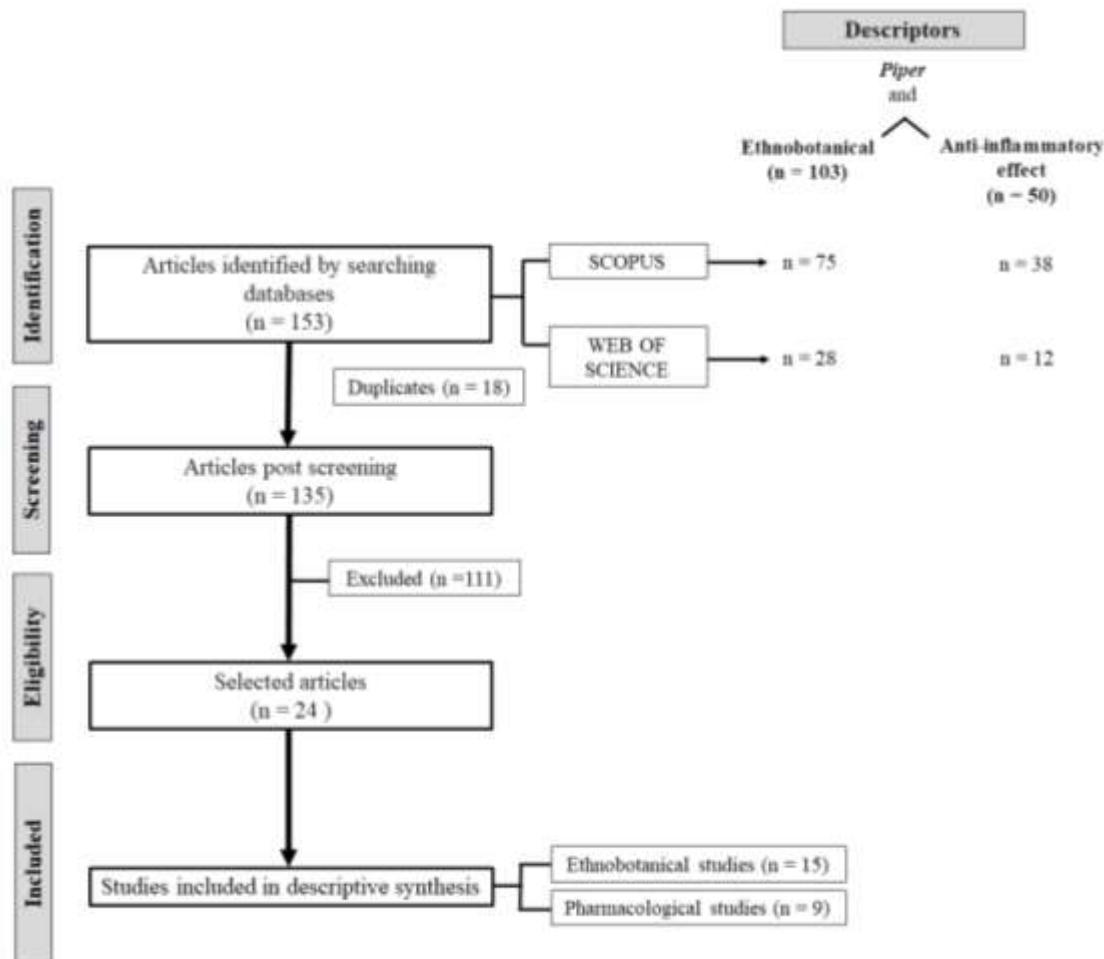
Figure 1. Selected research and results.

was not possible to carry out this analysis, once there is no validated tool for such finality.

SYRCLE's RoB tool was elaborated based on Cochrane Collaboration's (Higgins and Green, 2011) criteria, being adapted to evaluate the methodological quality and bias risks of experimental studies with non-human animals. This tool is composed of 10 entries that are related to 6 bias types: selection bias, performance bias, detection bias, friction bias, bias notification and other bias (Hooijmans et al., 2014). Based on the aforementioned tool, the studies were classified as "low bias risk", "high bias risk" and "not clear bias risk".

Extraction and data analysis

Data were obtained by one of the researchers (C.N.F.L.) using a list of selection criteria, which were accurately verified by the second researcher (L.F.L.). Regarding the articles that deal with ethnobotanical studies, the extracted items were information related to the species, the structure used, the form of use, traditional use, and the country. Regarding ethnopharmacological studies, the information obtained was: species, the structure used, type of extract/essential oil, type of test and effect, according to the literature. Grouping statistics (meta-analysis) were not possible due to methodological heterogeneity between studies.

RESULTS

In this systematic review, were found 153 articles in consulted databases. In the Scopus database, there were 113 articles and in Web of Science, 40 articles. After abstracts and titles screening were excluded 18 duplicated articles, remaining 135, and from full-text analysis, 111 texts were excluded. Finally, after inclusion and exclusion criteria application, 24 articles were selected for this study, in which 15 articles addressed ethnobotanical studies that demonstrated *Piper* species use in traditional medicine for inflammation and 9 articles with pharmacological studies of the species demonstrating elucidation for this activity (Figure 1).

Medicinal use of *Piper* genus species

Studies have demonstrated *Piper* genus use in traditional medicine. Here we highlight some ethnobotanical surveys that exhibit *Piper* species use for the treatment of

inflammatory diseases.

These species are widely used as a medicinal plant mainly in countries like Indonesia and India, in which a greater number of works were observed. The medicinal genus use occurs by the usage of all parts of the plant; leaves, roots, and fruits were the most used; and the forms of predominant uses in the studies were decoction, maceration, and powder use. However, some studies did not report both. About traditional species used for the treatment of symptoms caused by inflammation, inflammatory processes and inflammatory diseases, the prevalence of the treatment of respiratory diseases such as asthma and bronchitis was evidenced. Other medicinal uses are for rheumatism and gout, diseases considered inflammatory, and for the treatment of inflammatory processes such as wound healing, skin allergies and gastrointestinal ulcers. The symptoms of inflammation are fever and cough (Table 1).

Scientific elucidation of anti-inflammatory activity of *Piper* genus species

Related to the evaluation of the anti-inflammatory effect of the *Piper* genus, this systematic review showed that many species that already have medical use by population have not yet had their effects scientifically validated. According to research, only seven species have worked with their scientifically proven anti-inflammatory effect. The most commonly used plant part was leaves, the extract being the most predominant preparation form. Only two works are related to essential oil use. Related to the pharmacological test type used to evaluate the anti-inflammatory activity, paw edema, and pleurisy models were the most used (Table 2).

Methodological quality/bias risk

In Figures 2 and 3, all pre-clinical studies with non-humans animals were evaluated and classified as low bias risk (100%) based on the questions, if the evaluator did not have previous knowledge of results, if identification of incomplete results was adequate, if studies were free of selective results and if they were free of other problems that would cause bias.

DISCUSSION

Since primitive civilizations, man has been intimately related to plants in order to grow his own food and medicine. The popular medicinal use of plants is an ancient art based on the accumulation of information passed through successive generations (Zardo et al., 2016). Thus, ethnobotanical knowledge provides the pharmacological and industrial applications. From

popular wisdom about this type of plant that several research centers seek to prove the effectiveness of certain medicinal plants (Albuquerque, 2005).

In the present study, among the ethnobotanical studies reported for medicinal *Piper* genus use for inflammatory diseases, thirteen species were cited. In view of the analyzed studies, it can be seen that *Piper* species are used for different medicinal purposes and that often the vegetal part used, the form of use and medicinal application of a species can vary according to the cultural scene.

When comparing Tables 1 and 2 that demonstrate medicinal use and pharmacological elucidation, respectively, of this genus species, it can be verified that despite the report in the ethnobotanical studies few of these species had scientific elucidation confirmed in the area of the inflammation. The species that were scientifically elucidated and at the same time have reports of medicinal use by the population according to this systematic review were: *Piper umbellatum*, *Piper nigrum* and *Piper sarmentosum*.

People use *P. umbellatum* to treat fever and tissue cicatrization (Akendengue et al., 2005; Silalahi et al., 2015). Iwamoto et al. (2015), in their study, evaluated the anti-inflammatory activity of dichloromethane extract, from leaves of this species, on paw edema and carrageenan-induced peritonitis models; they observed decreased inflammation, and leukocyte migration.

P. nigrum is traditionally used to treat sore throat, fever, asthma, and cough (Albuquerque et al., 2007; Silalahi et al., 2015; Sureshkumar et al., 2017; Suroowan and Mahomoodally, 2016). Tasleem et al. (2014) elucidated the anti-inflammatory effect of this species in carrageenan-induced paw edema models that exhibited a significant decrease in edema at different concentrations of hexane and ethanolic extracts. Ahmed et al. (2013) demonstrated that the methanolic extract of this species significantly improves the cholinergic and characteristic neurodegeneration-induced dysfunction of Alzheimer's disease.

Ethnobotanical studies with *P. sarmentosum* reported the popular use for rheumatism and gout (Li et al., 2016) and its action for inflammation was elucidated by Lee et al. (2011) who demonstrated that the methanolic extract of this species reduced the production of nitric oxide (NO), which is one of the markers in one of the inflammation pathways (Gonçalves et al., 2000).

Kim et al. (2017) by means of tests aimed the inflammation ways of nitric oxide (NO) and prostaglandins (PGE_2) and the measurement of genes that trigger inflammatory processes; the methanolic extract of *P. attenuatum* exerted anti-inflammatory effects in *in vitro* tests with macrophage cells.

The other ten *Piper* species cited in Table 1 do not have a scientific elucidation approach to inflammation yet, according to databases used. The species are the following: *P. betle, P. marginatum, P. acutifolium, P.*

Table 1. Medicinal use of *Piper* species to treat symptoms of inflammation and inflammatory diseases.

Scientific name	Parts use	Method of preparation	Medicinal use	Country	Reference
Piper acutifolium Ruiz & Pav.	Leaves	Decoction	Gastritis	Peru	De Feo (2003)
	Leaves	Decoction and maceration	Ulcer	China	Li et al. (2016)
	Leaves	Liquid extract	Asthma	India	Savithramma et al. (2007)
	Leaves	NR	Febrifuge	Indonesia	Silalahi et al. (2015)
	Whole plant	Powder (taken orally)	Bronchitis and cough	India	Sureshkumar et al. (2017)
Piper betle L.	Leaves	Decoction	Sore throat Skinallergies	Indonesia	Sujarwo et al. (2015)
	Leaves	Juice (taken orally) Maceration (Used over the chest) Maceration (taken orally) Chewed	Cough Asthma Respiratory disorders Bronchitis	Africa	Suroowan and Mahomoodally (2016)
Piper cubeba Vahl.	Fruit	NR	Rheumatism	Indonesia	Silalahi et al. (2015)
Piper guineense Schumach. & Thonn.	Fruits Whole plant	Decoction Powder Balsam	Asthma	Africa	Gbekley et al. (2017)
Piper hancei Maxim.	Roots	Decoction	Gastritis, osteitis	China	Li et al. (2016)
Piper longum L.	Immature fruits and roots	Powder of roots mixed with honey (taken orally) Decoction Immature fruits and roots (taken orally)	Asthma	India	Savithramma et al. (2007)
	Fruit	NR	Rheumatism	Indonesia	Silalahi et al. (2015)
		Powder (taken orally)	Cough	India	Sureshkumar et al. (2017)
Piper marginatum Jacq.	Roots, leaves, stem	NR	Asthma	Brazil	Albuquerque et al. (2007)
Piper nigrum L.	Seeds	NR	Sore throat	Brazil	Albuquerque et al. (2007)
	Seeds	NR	Febrifuge	Indonesia	Silalahi et al. (2015)
	Roots	Powder (taken orally)	Asthma	India	Sureshkumar et al. (2017)
	Fruits	Powder (taken orally)	Cough	Africa	Suroowan and Mahomoodally (2016)
Piper peltatum L.	NR	NR	Skin problems, processes "granos"	Guatemala	Hitziger et al. (2016)
Piper sarmentosum Roxb.	Leaves and roots	Decoction and maceration	Rheumatism, gout	China	Li et al. (2016)
Piper tuerckheimii C.DC.	NR	NR	Fever and woundhealing	Guatemala	Hitziger et al. (2016)
	Leaves	NR	Inflammation, "loss of senses"	Guatemala	Michel et al. (2016)
Piper umbellatum L.	NR	Used on the skin	Febrifuge, Cicatrizant	Africa	Akendengue et al. (2005)
	Leaves	NR	Febrifuge	Indonesia	Silalahi et al. (2015)

NR, Not reported.
Source: Research data (2017).

*haucei, P. tuerckheimii, P. peltatum, P. longum, P. cubeba*e *P. guineense*. This shows the scarcity of studies that evaluate the anti-inflammatory activity of these species, but there are reports of genus

Table 2. Scientific elucidation of anti-inflammatory effect of species of the *Piper* genus.

Scientific name	Parts use	Type of extract/oil	Type of test	Effect	Reference
Piper aleyreanum C.DC.	Leaves	Essential oil	Pleurisy induced by carrageenan	The treatment with essential oil de *P. aleyreanum* reduced the main features of acute inflammation, including exudation and leukocyte number, mainly by a reduction in neutrophil differential cell count in the pleural cavity.	Lima et al. (2012)
				Oil essencial treatment (1-30 mg/kg, p.o.) caused a dose dependent reduction mainlyin ethanol-induced gastric lesions, decreasing the ulcer area mainly at doses of 10 and 30 mg/kg, with a mean ID50 value of 1.7 (0,9–3,1) mg/kg and an inhibition of 8774% at 10 mg/kg. Similarly, the essential oil via i.p. (10 mg/kg) reduced by 44,8712,8% gastric lesions induced by etanol.	
	Stem		Acute gastric lesions	The methanolic extract *P. attenuatum* Pa-ME Suppressed the Production of NO in macrophage stimulated with lipopolysaccharides, pam 3CSK4-and poly (I: C). The expression levels of mRNA and NO inducible synthetase (iNOS) and cyclooxygenase 2 (COX-2) were decreased.	
				It reduced the translocation of p50 / NF-κB and AP-1, as well as the activity of its enzymes Src, Syk and TAK1.An immunoprecipitation analysis was turned to the binding between its substrates which were induced by Src, Syk and TAK1 overexpression were also reduced. The methanolic extract of *P. attenuatum* exerts anti-inflammatory effectao by directing Src and Syk on the signaling path NF-κB and TAK1 on the signaling path AP-1.	
Piper attenuatum Buch.-Ham. Ex Miq.	NR	Methanolic extract	NO and PGE$_2$ Production Assay; Measurement of mRNA Expression Levels by Reverse- Transcriptase Polymerase Chain Reaction (RT-PCR).	The extract at the dose of 150 mg/kg decreases the TNF-a serum levels in Wistar rat with atherosclerosis by around 45.63%.	Kim et al. (2017)
Piper crocatum Ruiz & Pav.	Leaves	N-butanol	Decrease of TNF-α level and decrease of IL-6 levels pre and post-test	Inhibition of inflammatory mediators including TNF- α, IL-1, IL-6, and NO on LPS-induced macrophage cells.	Wahjuni et al. (2016)
P. nigrum L.	Leaves	Ethanolic extract	Pro-inflammatory activation of cells; Measurement of TNF-α, IL-β e IL-6; Concentration and inhibitory activity assay; Measurement of nitrite associated with NO concentration and inhibitory activity assay.	The hexane extract showed anti-inflammatory activity at a dose of 5 and 10 mg/kg compared to control, but less than the standard. The hexane extract exhibited maximum anti-inflammatory effect at a dose of 10 mg/kg after 60 min. The ethanol extract showed good anti-inflammatory activity at dose of 10 mg/kg as compared to control but less activity at all doses as compared to standard drug. The ethanol extract exhibited maximum activity at a dose of 10 mg/kg after 60 min.	Laksmitawati et al. (2017)
	Fruits	Ethanol extract and hexane	Carrageenan-Induced paw edema	The treatment with methanolic extract of *P. nigrum* L., only dose of 187.5 mg / kg reduced the serum AchE activity in the brain by 22.9%. However, both doses tested (187.5 mg/kg and 93.75 mg/kg) presented significance in CRP levels (-28.41% -25.90% 187.5 mg/kg b.w./-27.16%, -23,12% 93.75 mg/kg b.w.), NF-κB$_{65}$ (-37.86%, -17.52% 187.5 mg/kg b.w./-36,22%, -16,92% 93.75 mg/kg b.w.) e MCP-1, (-40.8%, -19.47% 187.5 mg/kg b.w./-35.57%, -17.27% 93.75 mg/kg b.w.). Thus, extract significantly improves the cholinergic dysfunction and neurodegeneration induced by AD inflammation.	Tasleem et al. (2014)

Table 2. Contd.

Piper sarmentosum Roxb.	Seeds	Methanolic extract	Experimental model of Alzheimer's disease induced by AlCl$_3$	The methanolic extract of *P. sarmentosum* (100, 50 and 25 µg / mL) reduced the production of nitric oxide in 62.82 ± 1.53, 46.53 ± 1.15 and 26.83 ± 1.73 respectively.	Ahmed et al. (2013)
	NR	Methanolic extract	Nitrite determination	*P. umbellatum* SDE treatment significantly inhibited the first phase of inflammation, in an independent way, as well as indomethacin 10 mg/kg. SDE was able to inhibit inflammation up to 4.5 hours, period coincident with prostaglandin release, which could suggest an action on prostaglandins production. In the second phase, all SDE doses inhibited inflammation at 48 hours while 400 mg/kg dose also inhibited the second inflammatory peak (72 h). This result suggests an effect on neutrophil mobilization, quite similar to the corticosteroids effects that efficiently inhibit the cellular phase of inflammation.	Lee et al. (2011)
				In the carrageenan-induced peritonitis model, leukocytes migration in the negative control group was 14160±1705 cells/mL and cell migration was inhibited both by dexamethasone (60.5%, 5 mg/kg) and by *P. umbellatum* SDE (52.0%, 200 mg/kg).	
Piper umbellatum L.	Leaves	Dichloromethane extract	Paw edema and pleurisy induced by carrageenan	The groups treated with oil essential of *Piper vicosanum* (OPV) doses of 100 and 300 mg/kg showed a significant decrease in edema at the four hours of observation. The inhibitions were 78±2% and 75±3% after 2 h and 80±2% and 86±3% after 4 h, respectively. Additionally, the oral administration of OPV significantly inhibited the leukocyte migration	Iwamoto et al. (2015)
				at all doses tested (100 and 300 mg/kg), with inhibitions of 70±3% and 85±2%, respectively, and higher inhibition at a dose of 300 mg/kg.	
Piper vicosanum C.DC.	Leaves	Essential oil	Paw edema and pleurisy induced by carrageenan		Brait et al. (2016)

NR, Not reported.
Source: Research data (2017).

use for inflammation treatment (Moreira et al., 1998; Felipe et al., 2006). In relation to the species *P. vicosanum*, *P. aleyreanum* and *P. crocatum* the opposite occurred; these have scientific elucidation and, nevertheless, were not found articles demonstrating the traditional use through ethnobotanical studies in the databases of Web of Science and Scopus.

Brait et al. (2015) studied *P. vicosanum* through models of paw edema and pleurisy induced by carrageenan; they obtained significant results for the edema reduction and for leukocyte migration in pleurisy. Also, *P. aleyreanum*, its anti-inflammatory effect also was evaluated by the carrageenan-induced pleurisy model and the acute gastric lesion test. Both tests were performed by Lima et al. (2012) and they obtained significant results regarding inflammatory processes reduction.

The studies cited above report the traditional use of this species, *P. aleyreanum*, as immunomodulatory, analgesic and antidepressant (Brait et al., 2015); people use it empirically in a more general way for bronchitis, intestinal pain, skin irritations and inflammation treatment (Lima et al., 2012).

Wahjuni et al. (2016) and Laksmitawati et al. (2017) performed tests with *P. crocatum*. Both evidenced the reduction of serum levels of TNF-α, IL-1, IL-6, and NO in Wistar rats and in LPS-induced macrophage cells, respectively. In these articles, authors do not describe empirical medicinal use; only bioactive components were present in these plants, correlating with its anti-inflammatory activity.

During the research were found several works that investigate the pharmacological activity of isolated compounds from the *Piper* genus for inflammation. Sheikh et al. (1993) isolated triterpenes and β-sitosterol from *P. betle* to plaquetary aggregation study and anti-inflammatory effect; the last one were used the model of paw edema induced by carrageenan to evaluate its potential.

In the species *P. nigrum* and *P. longum* the

Reference	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Kim et al. (2017).	+	+	?	+	?	-	+	+	+	+
Iwamoto et al. (2017).	?	+	-	-	-	-	+	+	+	+
Wahjune et al. (2016).	+	+	?	+	?	-	+	+	+	+
Brait et al. (2015).	?	+	?	?	-	-	+	+	+	+
Tasleem et al. (2014).	?	?	?	+	?	?	+	+	+	+
Ahmed et al. (2013).	+	+	?	+	-	-	+	+	+	+
Lima et al. (2012).	+	+	?	+	?	-	+	+	+	+
Lee et al. (2011).	+	+	?	+	?	-	+	+	+	+

+	Low risk of bias
-	High risk of bias
?	Unclear rik of bias

Figure 2. Methodological quality summary for preclinical trials: review of authors judgments about each methodological quality item for each study included: **P1,** Was the allocation sequence adequately generated and applied?; P2, Were the groups similar at baseline or were they adjusted for confounders in the analysis?; P3, Was the allocation to the different groups adequately concealed during?; P4, Were the animals randomly housed during the experiment?; P5, Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?; P6 - Were animals selected at random for outcome assessment?; P7, Was the outcome assessor blinded?; P8, Were incomplete outcome data adequately addressed?; P9, Are reports of the study free of selective outcome reporting?; P10, Was the study apparently free of other problems that could result in high risk of bias?).
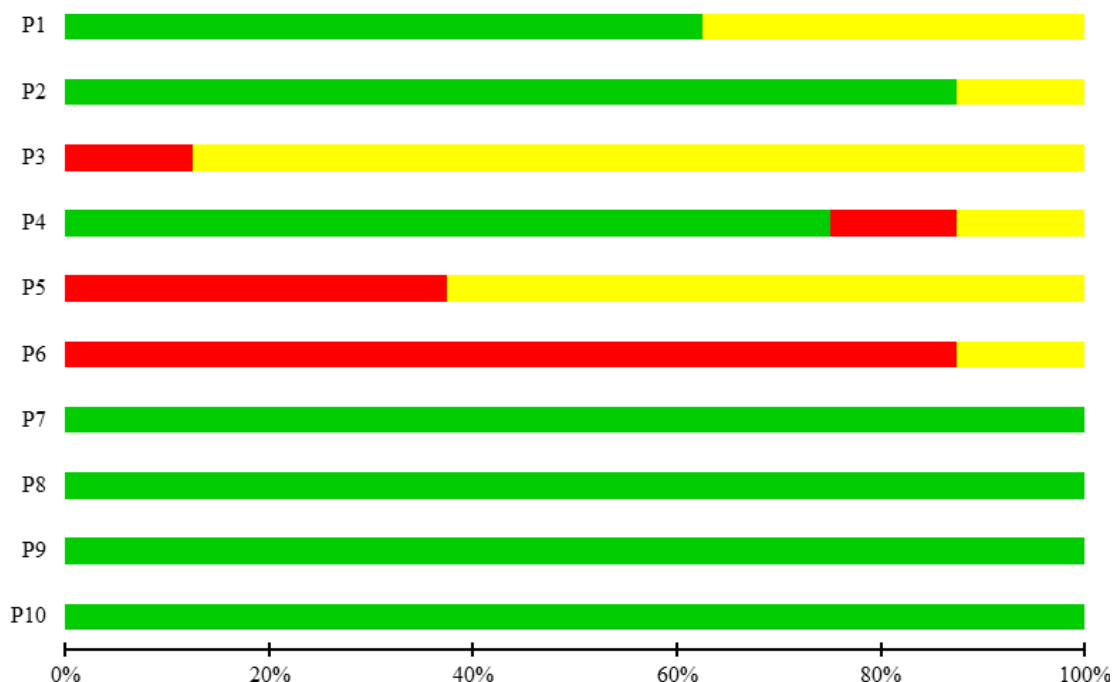
Figure 3. Methodological quality of included studies. Green bars indicate the proportion of articles that present low bias risk; red bars indicate the proportion of studies with high bias risk and yellow bars indicate the proportion of studies with unclear bias risk.

major component is piperine that has biological activities: anti-inflammatory, antioxidant, analgesic, healing, antifungal, antibacterial and facilitates blood circulation activity (Bae et al., 2011; Carnevallia and Araújo, 2015). Liang et al. (2016) evaluated piperine for pyroptosis in murine macrophages using pyroptosis assay. Cruz et al. (2013) studied its application in convulsion by checking deaths number and survivors through pilocarpine-induced seizure tests by the GABAergic system in an animal model. Kim et al. (2012) verified piperine inhibitory action on COX-1 with the COX-2 induction test by 13-acetate 12-myristate-frobol (PMA).

In Web of Science database were found five articles using isolated piperine compound and one with the compound allylpyrocatechol (APC) extracted from P. betle species. This component was evaluated by induced production of NO and PGE$_2$ of lipopolysaccharide (LPS) in murine macrophages cells to verify their anti-inflammatory capacity (Sarkar et al., 2008).

The remaining articles, using piperine, investigate: The apoptosis in neuronal cells and the anti-inflammatory activity in Parkinson's disease induced by 6-OHDA (Shrivastava et al., 2013), the neuro-inflammation on BV$_2$ microglia induced by inflammatory responses to LPS (Wang-Sheng et al., 2017), the ulcerative colitis by determining TLR4 receptor cholesterol in the pathway of cellular inflammation (Gupt et al., 2015) and the acute pancreatitis induced by cerulein by activation inhibition of mitogenic protein kinase (Bae et al., 2011).

Besides these works, other components such as: N-isobutylamine, (E) -β-caryophyllene (BCP) and other alkaloids were used, respectively, for anti-inflammatory effect of edema in formation in acute analgesia, in a murine model of nephropathy to verify anti-inflammatory properties and in neuroinflammatory processes in Parkinson's disease in the dopaminergic neuronal system (Reynoso-Moreno et al., 2017; Horváth et al., 2012; He et al., 2016).

Other works that approach isolated compounds, were by De León et al. (2002) who studied P. fimbriulatum through its furofan lignan (+)-diayangambin isolated component in vivo assays such as carrageenan-induced paw edema to verify its immunomodulatory and anti-inflammatory efficacy, and by Chion et al. (2003) which used piperlactam S isolated from P. kadsura for chemotaxis treatment by means of chemotactic migration, adhesion, measurement of phagocytic activity and cytokine production assays.

The analysis of the quality of methodology and the risk of bias of the studies inserted in a systematic review is of fundamental importance (Hooijmans et al., 2014). This is because this evaluation demonstrates that the research developed was well planned and carried out efficiently and that the results presented were interpreted correctly and comprehensible (Festing and Altman, 2002). In the articles addressed in this review, biases in the methodology were observed in preclinical studies with non-human animals, mainly in the allocation and blinding of the groups. This data require some special attention since studies that present a risk of methodological bias do not guarantee reliable results.

Conclusion

Studies covered in this systematic review prove that the Piper genus includes species with bioactive activities, with great potential for inflammatory disease treatment.

In traditional medicine, ethnobotanical studies showed that leaves, roots, and fruits were most used parts under decoction, maceration and powder form for inflammatory diseases of respiratory tract treatment, such as asthma, bronchitis, and cough. Already in the studies that scientifically validated Piper genus anti-inflammatory activity, only seven species presented works with their anti-inflammatory effect scientifically proven.

This review also showed that several ethnobotanical studies still present gaps in relation to detailed information regarding the plant part used and how they are used, as well as other data. The lack of such reports may lead to a loss for future pharmacological research. Thus, it is considered that interviews of this size should be very explanatory, aiming to collect as much information as possible about plant use in traditional medicine.

In the ethnopharmacological studies (in vivo or in vitro), there was also lack of information, mainly in relation to the plant part used to obtain the extract, implying that the lack of this data may be a reflection of the scarcity of ethnobotanical studies well structured. Another factor to be observed is that the pharmacological studies for inflammation with species use itself are still limited, with a larger number of works for isolated compounds from these plants. The research also did not present any study that reported genus use for clinical trials. In view of this, it can be seen that research with the Piper genus as a therapeutic agent in modern medicine is still very scarce, related to anti-inflammatory activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Journal of Medicinal Plants Research

Full Length Research Paper

In vitro antiplasmodial, cytotoxicity assay and partial chemical characterization of Kenyan *Physalis peruviana* L. (Solanaceae family) extracts

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Malaria is a protozoan infection of Public health concern with several new cases yearly reported. Control of malaria infections is constrained due to the toxicity of currently available drugs and the emergence of resistant malaria strains. The current study was designed to assess the antiplasmodial activity, cytotoxicity and to partially characterize Kenyan *Physalis peruviana* extracts in order to determine their utility as a possible source of a new antimalarial drug. Antiplasmodial activity of *P. peruviana* extracts was evaluated *in vitro* using *Plasmodium falciparum* D6 chloroquine-sensitive, and W2 chloroquine-resistant by semi-automated microdilution technique. Cytotoxicity assay was determined using Vero cells; while partial characterization determined using Fourier transformer infra-red spectrophotometer (FTIR) and Gas chromatography-mass spectrophotometer (GC-MS). The antiplasmodial activity (IC_{50}) of *P. peruviana* extracts against chloroquine-sensitive (D6) *P. falciparum* strain ranged from 14.719±0.744 to >100 ug/ml. For W2, strain antiplasmodial activity ranged from 8.303±1.062 to >100 ug/ml. All the FTIR and GC-MS analysis of *P. peruviana* leave extract revealed the presence of biologically active components. There is a need for further studies using purified extracts as a means of coming up with possible novel antiplasmodial drugs. *P. peruviana* extracts were not toxic to Vero cells.

Key words: Antiplasmodial, cytotoxicity, Vero cells, *Physalis peruviana* extracts.

INTRODUCTION

Malaria is one of the most severe life-threatening protozoan diseases typically characterized by fever, paroxysm and flu-like symptoms recurring within 48 to 72 h cycles (Njokah et al., 2016). *Plasmodium falciparum* is by far the most virulent species of parasites that affect humans. In the 2017 World Health Organization (WHO) report on malaria, 216 million episodes of malaria and 445,000 deaths were reported, with 90% of cases and

91% of deaths affecting the WHO African region (Kweyamba et al., 2019). Eradication of malaria has become a serious challenge due to the emergence of *P. falciparum* species resistant to the most potent recently developed antimalarial drugs, such as artemisinin-based combinations (WHO, 2007). As a result, there are limited options for the management of patients infected with such resistant parasites, a situation that necessitates renewed efforts to identify new chemical compounds with antimalarial activity.

Physalis peruviana L. belongs to the Solanaceae family. Botanically, *P. peruviana* can be classified as belonging to the Plantae Kingdom, the order solanales, the family Solanaceae, subfamily of solanoideae, tribe of physaleae, subtribe of physalinae and the species of *Physalis peruviana* (Sharma et al., 2015). The *P. peruviana* grown in Kenya is thought to have probable origin from tropical South America (Maundu et al., 1999) and is well adapted to Kenyan local environmental conditions. It is commonly found growing in the wild in Kenyan forests and in infertile soils where its fruits are collected and eaten. In Kenya, *P. peruviana* was reported to be used in herbal medicine against asthma (Njoroge and Bussmann, 2006), while in the Kagera region northwestern Tanzania, the fruit juice of *P. peruviana* was used as a cure for malaria (Moshi et al., 2012).

In the current study, the antiplasmodial activity and cytotoxicity of *P. peruviana* extracts were investigated. In addition, partial characterization of *P. peruviana* L. extracts was carried out with the aim of identifying the active compounds in the crude extracts with the highest anti-plasmodial activity.

MATERIALS AND METHODS

Study site, collection of *P. peruviana* and authentication

The *P. peruviana* plant materials were collected in February 2013 from Nyeri County [0°25`0``South, 36°57`0``East] located in the Central part of Kenya. Nyeri is 162 Km North of Nairobi. This area is known to have good reserves of *P. peruviana* L. The whole plant materials were collected in 2013 and identified by National Museums of Kenya Botanists and a Voucher specimen number EAH001PK deposited at the National Museums of Kenya Herbarium, Nairobi. Various parts; fruits, leaves, stem, and roots were separated, dried under shade and pulverized in a hammer mill fitted with a sieve of 0.5mm pores.

Antiplasmodial and cytotoxicity studies were carried out at the Centre for Biotechnology Research and Development (CBRD) Malaria Laboratory, Kenya Medical Research Institute (KEMRI) Headquarters, Nairobi, Kenya. Partial characterization of dichloromethane *P. peruviana* leaves extract was carried out at Jomo Kenyatta University of Agriculture and Technology chemistry laboratory, Nairobi, Kenya.

Preparation of *P. peruviana* aqueous, methanolic, and dichloromethane extracts

Preparation of *P. peruviana* extracts was carried out using methods described by Ubulom et al. (2011). Potions of pulverized plant parts were soaked separately in distilled water, methanol and dichloromethane, for 72 h with stirring at regular intervals. The extracts were repeatedly filtered using a sterile Whatman No. 1 filter paper (Jaca and Kambizi, 2011). The aqueous filtrates were freeze-dried, while the methanol and dichloromethane extracts were concentrated under vacuum at 40°C in a Buchii rotary evaporator.

The percentage yield was determined using the method used by Ogila (2010) as follows:

Percentage yield = (Weight of extract/Weight of ground material) X 100.

For identification purposes the extracts were assigned codes as follows; APPL = aqueous extracts of *P. peruviana* leaf; APPS = aqueous extracts of *P. peruviana* stem; APPF = aqueous extracts of *P. peruviana* fruit, APPR = aqueous extracts of *P. peruviana* root; MPPL = methanolic extracts of *Physalis peruviana* L leaves (MPPL), MPPS = methanolic extracts of *Physalis peruviana* L stem (MPPS), MPPF = methanolic extracts of *Physalis peruviana* L fruit (MPPF), MPPR = methanolic extracts of *Physalis peruviana* L root (MPPR), DPPL = dichloromethane extracts of *P. peruviana* leaf; DPPS = dichloromethane extracts of *P. peruviana* stem; DPPF = dichloromethane extracts of *P. peruviana* fruit; DPPR = dichloromethane extracts of *P. peruviana* root. All the extracts were kept desiccated at 4°C until use.

In vitro antiplasmodial bioassay

P. peruviana extracts were assayed using an automated microdilution technique to determine 50% growth inhibition of cultured parasites (Chulay et al., 1983; Desjardins et al., 1979).

Two different clones of *P. falciparum* were used in this study. The chloroquine-sensitive Sierra Leone 1 (D6) and chloroquine-resistant Indochina 1 (W2) strains were grown in a continuous culture supplemented with mixed gas (92% nitrogen, 5% carbon dioxide and 3% oxygen), 10% human serum and 6% hematocrit of O+ red blood cells and 5-fluorocytosine. Once cultures reached the optimum growth; growth rate ≥ 3.0% and parasitemia of > 3 % with at least an 80% ring developmental stage present, parasite solution was transferred to a 96 well microtiter plate with wells pre-coated with the test sample. The test samples were serially diluted across the plate to provide a range of concentrations used to accurately determine IC_{50} values. Plates were incubated in a gas chamber for 48 h after which ^{3}H-hypoxanthine was added and parasites allowed to grow for a maximum of 18 h. Cells were processed with a 96 well plate harvester (MicroBeta) onto filter mat paper (Wallac) and washed to eliminate unincorporated isotope. Filters were measured for *P. peruviana* antiplasmodial activity in a micro-titer plate scintillation counter (Wallac). Data from the counter was processed using the Oracle database program to determine IC_{50} values.

Cytotoxicity assays

The cellular cytotoxicity assay was used to test the cytotoxicity effects of individual extracts as was described by Wabwoba (2010) with slight modification. Briefly, Vero cells were seeded in minimum essential medium (MEM) (ATTC® 30-200™) supplemented with 10% Fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in 25 ml cell culture flasks and incubated for 24 h at 37°C in 5% CO_2 humified atmosphere.

The Vero cells were harvested by trypsinization and pooled in 50 ml vials from which 100 µl suspensions at a concentration of 1 x 10^6 cells was added into 2 wells of rows A-H of a 96-well flat bottomed microtiter plate. A 100 µl of each *P. peruviana* extract was added and the plates incubated at 37°C in 5% CO_2 atmosphere. Cells without extracts and medium alone served as the controls. A 100 µl

Table 1. Percentage yields of aqueous, methanolic and dichloromethane *P. peruviana* extracts.

Plant extract	Amount of ground part in (g)	The yield of extract (g)	The yield of the extract (%)
APPL	100	10	10
APPS	100	3	3
APPF	120	0.6	0.5
APPR	100	6	6
MPPL	40	3.4	8.5
MPPS	100	5	5
MPPF	40	0.4	0.8
MPPR	100	5	5
DPPL	40	1	2.5
DPPS	40	1.34	3.4
DPPF	120	0.83	0.7
DPPR	120	4.3	3.6

of the cell suspension was discarded from each well followed with an addition of 10 µl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Roche, Indianapolis USA). After 4 h of incubation, the medium together with MTT was aspirated off from the wells, 100 µl of Dimethylsulphoxide added and plates shaken for 5 min. The absorbance was measured for each well at 562 nm using a microtiter plate reader (Wang et al., 2006).

Partial characterization of dichloromethane *P. peruviana* leaves extract by Fourier Transform infrared spectrophotometer (FTIR) and gas chromatography-mass spectrophotometry (GC-MS)

Fourier Transform Infrared Spectrophotometer (FTIR)

About 0.02 g of dried DPPL extract was reconstituted in dichloromethane and a drop of the extract mounted on NaCl plates for the FTIR analysis. The analysis was carried out using a Shimadzu 8400 FTIR with the scan ranging from 400 - 4000 cm^{-1}. The scanning was made through the IR region and the *P.peruviana* dichloromethane leaves extract functional groups determined according to the method described by Maobe and Nyarango (2013).

Gas chromatography-mass spectrometry analysis of dichloromethane leaves extract

Initially, 0.05 g of a homogenized DPPL extract was dissolved in 5 ml of dichloromethane in a centrifuge tube. The mixture was vortexed for 10 s and sonicated for 10 min. The mixture was centrifuged at 15000 *g* for 5 min. The supernatant was filtered before analysis in the GC-MS. Samples were analyzed using an Agilent 7890 Gas Chromatograph coupled to an Agilent 5975 Mass Spectrometer. The column used was the HP 5 MS capillary column: 30 m; 0.25 mm ID; 0.25 µm film thickness. The oven temperature program was initially set at 35°C and held for 3 min. It was then raised to 285°C at a rate of 10°C/ min. The oven was maintained at this temperature for 23 minutes. The total run time was 50 minutes. Mass spectrometry was done in full scan mode from 40 – 500 m/z with a solvent delay time of 3.5 minutes. Carrier gas was He (99.999%) at a flow rate of 1 ml/minute. One µL of the sample was injected using a split/splitless injector. The injector temperature was set at 200°C and the interface temperature was set at 280°C

according to the method described by Wamalwa et al., (2015).

Statistical analysis

An independent t-test was performed on inhibition (IC_{50} in µg/ml) of *P. peruviana* extracts to both chloroquine-sensitive Sierra Leone 1 (D6) and chloroquine-resistant Indochina 1 (W2) strains. The level of significance was fixed at $P < 0.05$.

Ethical considerations

Permission to carry out the study was granted by the Kenya Medical Research Institute Ethical and Scientific Steering Committees (reference numbers KEMRI/RES/7/3/1 and ESACIPAC/SSC/101472 respectively).

RESULTS

Percentage yields of various *P. peruviana* extracts

The percentage yields for aqueous, methanolic, and dichloromethane *P. peruviana* extracts ranged from 0.5 to 10.0% (Table 1). *P. peruviana* aqueous leaves extract had the highest percentage yield, while the lowest percentage yield was recorded in the aqueous fruit extract.

Antiplasmodial assay of *P. peruviana* extracts

Most of the *P. peruviana* extracts exhibited various antiplasmodial activities. According to Rasoanaivo et al. (1992), antimalarial activity of plants is described as very active: IC_{50} <5 µg/ml, active: IC_{50} greater than 5 µg/ml but less than 50 µg/ml, weakly active: IC_{50} value greater than 50 µg/ml but less than 100 µg/ml and inactive: IC_{50} > 100 µg/ml. In this analysis, APPF and MPPF were the only ones found to be inactive (Table 2). An independent t-test

Table 2. *Physalis peruviana* aqueous, methanolic and dichloromethane extracts antiplasmodial activity (IC_{50}).

Extract	D6 IC_{50} (µg/ml)	W2 IC_{50} (µg/ml)
APPL	39.984±3.797	29.522±15.752
APPS	48.664±2.535	40.594±1.194
APPF	>100	>100
APPR	58.93±0.899	61.333±5.388
MPPL	36.479±1.519	20.855±6.044
MPPS	28.417±0.417	39.673±0.998
MPPF	>100 µg/ml	>100 µg/ml
MPPR	46.403±4.267	58.072±9.85
DPPL	14.719±0.744	8.303±1.062
DPPS	29.505±0.213	16.757±6.609
DPPF	66.752±1.098	32.945±18.282
DPPR	62.345±0.845	22.806±9.97
Chloroquine (ng/ml)	12.67±0.03	127.66±1.96

The IC_{50} values are represented as mean±SEM *P.peruviana* aqueous, methanol, dichloromethane extracts, and Chloroquine control drug against W2 and D6 *Plasmodium falciparum* strains.
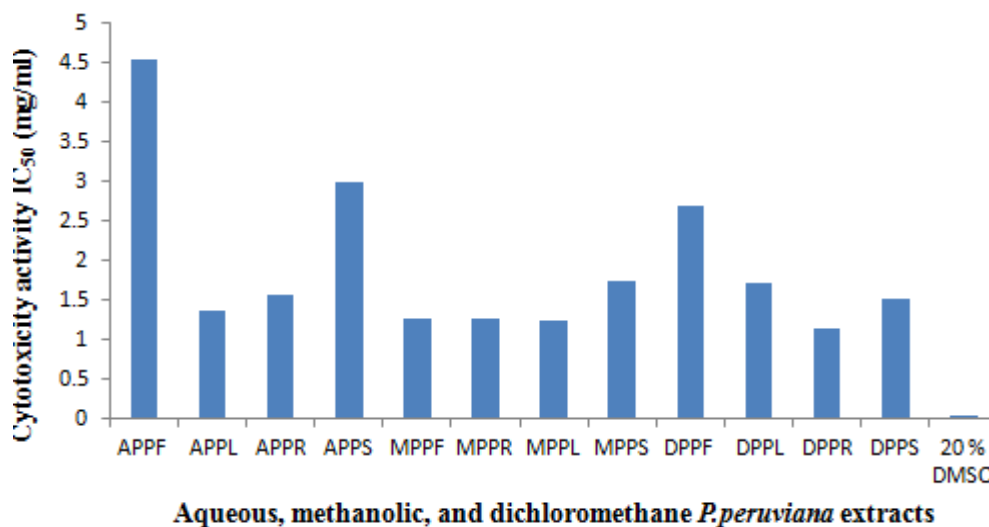
Figure 1. Cytotoxicity effects of aqueous, methanolic and dichloromethane *P. peruviana* extracts using Vero cells.

was performed to test the inhibition activity of the plant extracts against chloroquine-sensitive (D6) and chloroquine-resistant (W2) *P. falciparum* isolates. The inhibition activity of the extracts against D6 and W2 IC_{50} (µg/ml), t (22) = 0.016; P = 0.988, was not statistically different (P > 0.05).

Cytotoxicity effects of *P. peruviana* extracts

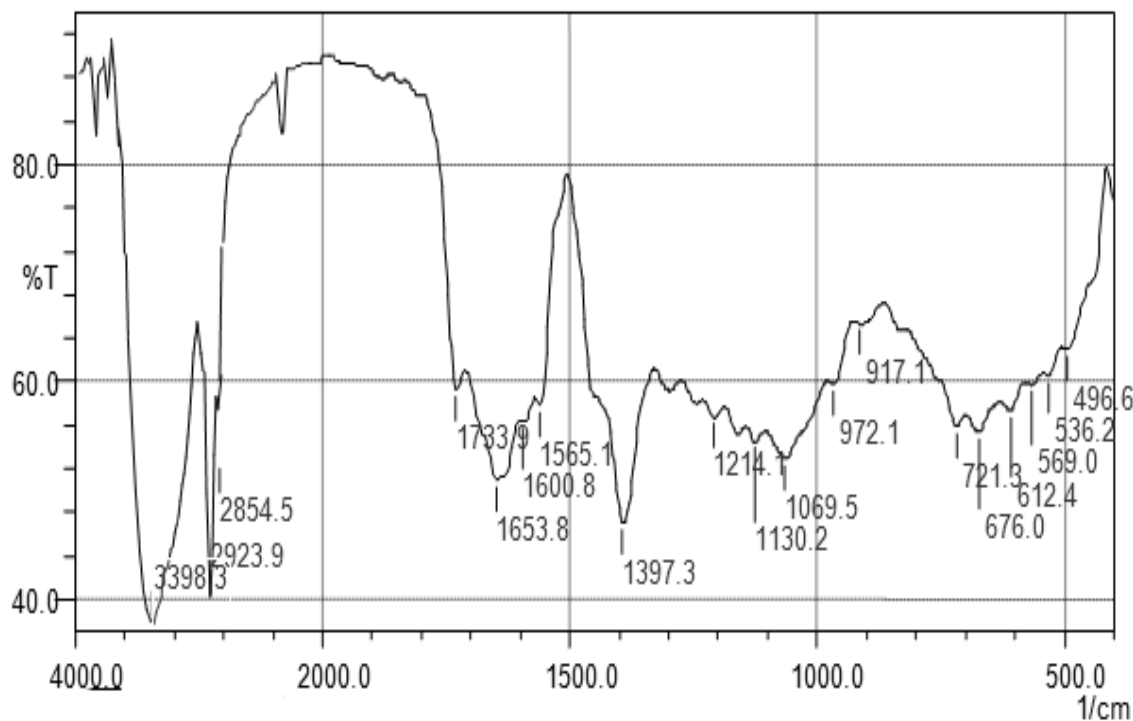All the *P. peruviana* extracts were less toxic to Vero cells

(higher IC_{50} values) as compared to the control, 20% DMSO (Figure 1).

Partial characterization of dichloromethane *P. peruviana* leaves extract FTIR and GC-MS

Fourier Transform Infrared Spectrophotometer (FTIR) analysis

The spectrum obtained indicated an intense absorbance

Figure 2. FTIR spectra of *P. peruviana* dichloromethane leaf extracts showing absorbance peaks.

at 3398 cm^{-1} attributed to O-H stretch vibrations in alcohol (Nithyadevi and Sivakumar, 2015). Polyphenols present in the plant extract is attributable to this absorbance value. The sharp shoulder peaks at 2923.9 and 2854.5 cm^{-1} are characteristic of C-H stretch in alkanes (Maobe and Nyarango, 2013). The small absorbance peak at 1733.9 cm^{-1} is attributed to the carbonyl C=O stretch in carboxylic acids, while the carbonyl stretch in ketones could be responsible for the absorbance peak at 1653.8 cm^{-1}. The absorbance peak at 1565.1 cm^{-1} due to N-H bending and peaks at 1130.2 and 1069.5 cm^{-1} attributed to C-N stretch vibrations in aliphatic amines could indicate the presence of alkaloids in the sample (Figure 2).

Gas chromatography-mass spectrometry analysis of *P. peruviana* dichloromethane leaves extract

Gas chromatography-mass spectrometry analysis of *P. peruviana* dichloromethane leave extracts revealed 8 phytochemicals with molecular weight ranges of 256 to 436 daltons. These phytochemicals included: Hexahydrofarnesyl acetone, n- Hexadecanoic acid, phytol, linoleic acid, ethyl iso-allocholate, vitamin E, campesterol and stigmasterol (Table 3).

The 8 Phytochemicals in Table 3 are also presented in the form of total ion current (TIC) (Figure 3).

DISCUSSION

The objective of the current study was to evaluate the antiplasmodial activity and safety of aqueous, methanolic and dichloromethane *P. peruviana* extracts, and also to partially characterize the extract exhibiting the highest antiplasmodial activity.

The yield of aqueous, methanol and dichloromethane *P. peruviana* L. extracts ranged from 0.5 - 10, 0.8 - 8.5 and 0.7 - 3.6% respectively. These results were comparable to those of other researchers who reported that the more polar solvents yielded greater quantities of extracts (Gaba et al., 2019; Abubakar, 2010).

From this study 22 (91.7%) out of the 24 *P. peruviana* extracts exhibited antiplasmodial activity against the two laboratory *P. falciparum* clones, chloroquine-sensitive D6 (Sierra Leone) and chloroquine-resistant W2 (Indochina). The mean IC$_{50}$ (μg/ml) for the antiplasmodial activity for *P. peruviana* extracts against the D6 clone ranged from 14.719±0.744 to > 100 μg/ml, while the mean IC$_{50}$ of *P. peruviana* extracts ranged from 8.303±1.062 to > 100 μg/ml. The DPPS extract exhibited the highest activity for both clones while the weakest antiplasmodial activity was from APPF and MPPF both at > 100 μg/ml. According to Rasaonaivo (1992), the classification of antiplasmodial activity on crude plant extracts, APPF and MPPF could, therefore, be classified as inactive. These results are in agreement with a study carried out by Moshi (2012) in the Kagera region, northwestern Tanzania which found that

Table 3. Phytochemicals of *P. peruviana* dichloromethane leaves extract by GC-MS analysis.

Peak number in the sample TIC	Retention time (min)	Compound name	Molecular weight (Daltons)	Reported Activity	References/ literature
2	22.635	Hexahydrofarnesyl acetone	268	Biologically active	Nikkhah et al. (2017)
3	24.180	n-Hexadecanoic acid	256	Antioxidant, anticancer	Nikkhah et al. (2017)
4	25.323	Phytol	296	Antimicrobial, anti-inflammatory, Anticancer, diuretic	Nibret and Wink (2010)
5	25.905	Linolenic acid	278	Cytotoxic, antitrypanosomal	Nikkhah et al. (2017)
6	27.518	Ethyl iso-allocholate	436	Antifungal	Mohan et al. (2012)
7	35.289	Vitamin E	430	Antioxidant	Malathi et al. (2016)
8	37.126	Campesterol	400	Anti-CDV	Choe and Min (2009)
9	37.776	Stigmasterol	412	Anti-osteoathritic	Genser et al. (2012)
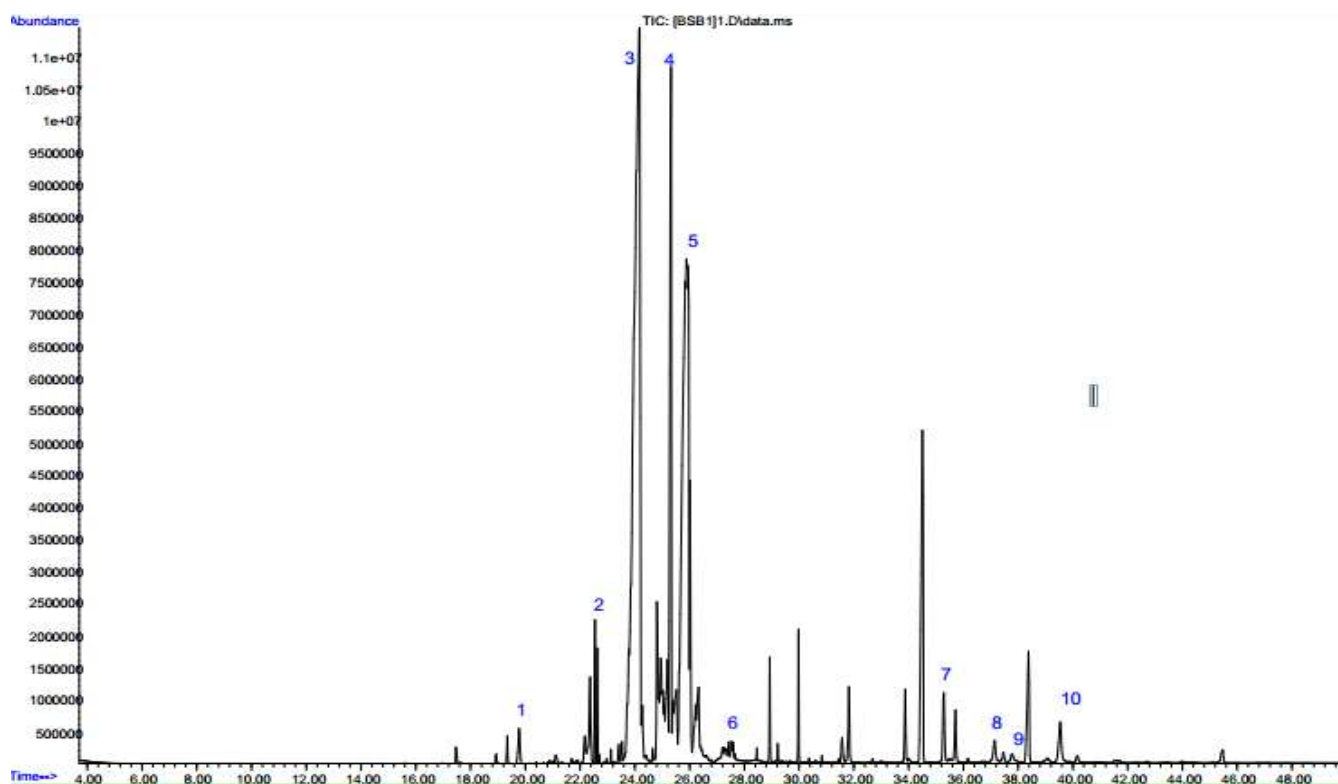
Figure 3. Total Ion Current (TIC) for *P. peruviana* leaves dichloromethane extract.

P. peruviana was being used to treat malaria as herbal medicine. The results are comparable to an *in vitro* study carried out by N'guessan et al. (2010) on *P. angulata* a similar species to *P. Physalis* that exhibited antiplasmodial falciparum activity of IC_{50} (µg/ml) of 7.9±0.3. These results are quite close to antiplasmodial activity exhibited by dichloromethane *Physalis peruviana* L extracts in the current study.

It was necessary to determine the safety profile of plant extracts due to the complexity and natural biological variations emanating from them (Cowan, 1999). Cytotoxicity effects of *P. peruviana* extracts using Vero cells revealed that all extracts were less toxic compared to the control. However, according to the US National Cancer Institute (NCI), *P. peruviana* extracts tested were not within the toxic group of $IC_{50} < 20$ µg/ml in the preliminary assay (Nathyadevi and Sivakumar, 2015).

The FTIR is an invaluable tool for the characterization

and identification of phytochemicals or functional groups present in an unknown mixture of plant extract (Maobe and Nyarango, 2013). The spectrum obtained indicated the presence of O-H stretching for alcohols, Polyphenols, C-H stretch for alkanes, the carbonyl C=O stretch for carboxylic acids, and the carbonyl stretching for ketones, N-H bending and peaks at 1130.2 cm^{-1} and 1069.5 cm^{-1} attributed to C-N stretch vibrations in aliphatic amines could indicate the presence of alkaloids in the sample. The presence of these functional groups could be attributed to the medicinal properties as was revealed from a previous study (Maobe and Nyarango, 2013).

Gas chromatography-mass spectrometry revealed the presence of some compounds with biological activities such as Hexahydrofarnesyl acetone, n-Hexadecanoic acid, Phytol, Linolenic acid, Ethyl iso-allocholate, vitamin E, campesterol and stigmasterol. Linoleic acid, Phytol and Ethyl isoallocholate have been associated with antimicrobial activities (Nibret and Wink, 2010; Mohan et al., 2012; Malathi et al., 2016), which could have been attributed to the antiplasmodial activity of *P.peruviana* extracts. In a study carried out by Kamau et al. (2017), *P. peruviana* extracts were found to contain biologically active compounds such as tannins, saponins, steroids flavonoids, and alkaloids. Alkaloids have been associated with the antiplasmodial activity (Wright and Philipson, 1990).

Conclusion

Aqueous, methanolic and dichloromethane *P. peruviana* leaves, stem, fruits, and roots extract exhibit antiplasmodial activity against *P. falciparum*. The ability of 92% of *P. peruviana* extracts to kill chloroquine-resistant Indochina 1 (W2) strains is of importance where chloroquine-resistant malaria is in existence. The investigations demonstrated the effectiveness and safety of using herbal treatment of malarial by *P. peruviana* by the rural indigenous communities. Partial characterization of *P. peruviana* extracts by FTIR and GC-MS revealed biologically active phytochemicals that could be attributed to the antiplasmodial activity exhibited by *P. peruviana* extracts. The study recommends the use of purified extracts and elucidation of actual compounds against *P. falciparum* activity.

ACKNOWLEDGMENTS

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Journal of Medicinal Plants Research

Full Length Research Paper

Quantification of some phenolics acids and flavonoids in *Cola nitida, Garcinia kola* and *Buchholzia coriacea* using high performance liquid chromatography- diode array detection (HPLC-DAD)

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Kola seeds are highly valued in most African communities due to their medicinal benefits and socio-cultural application during ceremonies. Apart from their neuro-stimulatory effect, the other health benefits such as antioxidant activities which are closely associated with the level of phenolic constituents have also been widely reported. The main purpose of this study is to determine the quantity of quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid in *Cola nitida, Garcinia kola and Buchholzia coriacea* using HPLC-DAD. Ethanolic extracts of the selected kola plants was obtained by cold maceration and analysed by HPLC-DAD in accordance with standard methods. The regression coefficient (r^2) from the calibration curve for caffeic acid = 0.999, chlorogenic acid = 0.998, gallic acid = 0.999, ferulic acid = 0.998, quercetin = 0.996 and rutin = 0.997. The repeatability gave % RSD of 0.6, 1.63, 0.44, 1.55, 3.65 and 4.67 for caffeic acid, chlorogenic acid, gallic acid, ferulic acid, quercetin and rutin respectively. The quantity of these compounds in *C. nitida* was caffeic acid (101.24 mg/g), chlorogenic acid (36.35 mg/g), gallic acid (16.99 mg/g) ferulic acid (1.47 mg/g) while quercetin and rutin were not detected. In *Garcinia kola*, caffeic acid was (0.84 mg/g), gallic acid (1.02 mg/g), ferulic acid (21.83 mg/g), quercetin (53.24 mg/g), rutin (0.49 mg/g) and chlorogenic acid was not detected. *B. coriaceae* had caffeic acid (1.03 mg/g), chlorogenic acid (0.33 mg/g), gallic acid (1.07 mg/g) while ferulic acid, quercetin and rutin were not detected. Using this analytical method, the quantities of some phenolics and flavonoids compounds were determined, and the most abundant compound in the three species of kola was caffeic acid in *C. nitida* and quercetin in *Garcinia kola*. This study also showed that *C. nitida* contains high amounts of phenolics compounds as compare to the other species of kola seeds investigated in the study.

Key word: Phenolic acids, flavonoids, high-performance liquid chromatography- diode-array detector (HPLC-DAD), *Cola nitida, Garcinia cola, Buchholzia coriacea*.

INTRODUCTION

Cola nitida (kola nut) belongs to the plant family sterculiaceae, it grows as a tree. It is believed that kola nut originates from Ghana and Ivory Coast. There are over fifty species of Kola plant but only seven have edible nuts (Okoli et al., 2012). Kola nut contain large amount of caffeine and threobromine hence, its use as stimulant.

Kola nut generate a strong sense of euphoria and well-being, enhances alertness and physical energy, its elevate mood, increase tactile sensitivity, restrain appetite and hunger; it is also use as aphrodisiac. Kola nut is used to treat whooping cough and asthma because the caffeine present in it acts as a bronchodilator expanding the bronchial air passage (Odebunmi et al., 2009).

Garcinia cola (bitter kola) also called wonder nut belongs to the family Clusiacea/Guttiferae. It grows mostly in coastal rain forest in south Western and south Eastern part of Nigeria. Bitter kola also contains caffeine and threebromine. It is also an aphrodisiac. It is believed that bitter kola cleans the digestive system, without side effect; hence, it is use in the treatment of abdominal problems (Odebunmi et al., 2009). Bitter kola contains flavonoids and other phenolics compounds. It is used as an anti-inflammatory agent; it also serves as raw material in pharmaceutical industries (Mazi et al., 2013).

Buchholzia coriacea (wonderful kola) was named after R.W Buchholz who first collected the plant in Cameroon in the late 19th century. The seeds of the plant earn it a common name "wonderful kola" because of its wide usage in traditional medicine. It is an evergreen, small to medium-sized tree growing up to 20 m tall (Erhirhie et al., 2015). It belongs to the family capparaceae juss. It grows mostly in Cameroon, Ivory Coast and other part of the world. Wonderful kola has numerous medicinal benefits and each part of the plant is use for treatment of ailments and diseases such as ulcer, hypertension, diabetes, malaria, cough to mention but a few (Owonikoko et al., 2015).

Seeds of kola nut (*Cola nitida*), bitter kola (*Garcinia cola*) and wonderful kola *(B. coriacea)* are well appreciated in Africa, for their socio-cultural, nutritive and medicinal values. However, these three species of kola has numerous pharmacological activities such as antioxidant, anti-cancer, anti-diabetic, anti-inflammatory, anti-hypertensive, anti-viral, cardiovascular activity, antimicrobial just to mention but a few (Zailani et al., 2016; Buba et al., 2016; Izah et al., 2018). And the numerous health benefits of these plants is due to the amount of important chemical compounds found in them such as phenolic acids, flavonoids and others (Duru et al., 2012; Ibrahim and Fagbohun, 2014). Some of these phenolic acids include caffeic acid, chlorogenic acid, gallic acid, ferulic acid and the flavonoids are Quercetin and Rutin. In a previous study, (Nyamien et al., 2017) reported that cola nitida (kola nut), contain caffeic acid, chlorogenic acid and others compounds, while quercetin and other compounds were reported in *G. kola (Bitter kola)* (Buba et al., 2016). However, there is paucity of

data on the presence of these phenolics in *B. coriacea* (wonderful kola), secondly the amount of these compounds present in each of this kola are not documented. This study is design to quantify quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid in *C. nitida, G. kola* and *B. coriacea* to determine the amount of these compounds presents in each of the species and to compare the quantity of the compounds among the species using a high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Sample collection, identification and preparation

The seeds of *C. nitida, G. kola and B. Coriacea* were purchased from Karmo market in Abuja, Federal capital territory, Nigeria. The seeds were identified and authenticated in the Herbarium unit, department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria. The nuts were sliced into pieces to enhance drying. The sliced pieces were air dried for two weeks and crushed into powder using mortar and pestle.

Preparation of extract solutions

The dried powder seeds of *C. nitida* (26.0895 g)*, G. kola* (25.3748 g) and *B. coriacea* (24.0872 g) were macerated with 100 mL of absolute ethanol each for the period of 48 hours. The sample solutions were filtered through Whatmann No. 1 filter paper. After which filtrate was transferred into a clean beaker and allow to evaporate to dry at room temperature. 0.2 g of the dried extracts was accurately weighed, transferred in to a volumetric flask and made up with 10 mL absolute ethanol. The final solution was passed through a 0.45 µm membrane filter prior to use. An aliquot of 10 µl of each sample solution was injected into the HPLC system for analysis.

Preparation of standard solutions

Reference compounds of quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid were all purchased from Sigma-Aldrich Germany. Suitable amount of the six reference compounds were accurately weighed, then dissolved in ethanol. The mixed stock solution contained 500 µg/ml of caffeic acid, 250 µg/ml of chlorogenic acid, 250 µg/ml of gallic acid, 250 µg/ml of quercetin, 500 µg/ml of rutin and 550 µg/ml of ferulic acid. After filtered, the reference substance solution was directly injected into the HPLC.

Apparatus and chromatographic conditions

The HPLC analysis was carried out on a Shimadzu HPLC system comprising Ultra-Fast LC-20AB prominence equipped with SIL-20AC autosampler; DGU-20A3 degasser; SPDM20A UV-diode array detector (UV-DAD); column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software

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Table 1. The calibration curve.

Compound	Linear equation	Correlation coefficient (R^2)	Linear range (µg/ml)
Caffeic acid	$Y = 11103x-15442$	0.999	25-500
Chlorogenic acid	$Y =5586.5x+196030$	0.998	7.812-250
Gallic acid	$Y = 9248.1x+39417$	0.999	10-250
Ferulic acid	$Y = 6301x+29944$	0.998	10-550
Quercetin	$Y = 12819x+37596$	0.996	60-250
Rutin	$Y = 7892x+67898$	0.997	3-500

Table 2. Precision and reproducibility.

Compound	Precision n=6		Repeatability n=6	
	Concentration (µg/ml)	%RSD	Concentration (µg/ml)	%RSD
Caffeic acid	500	0.64	500	0.77
Chlorogenic acid	500	1.63	500	1.05
Gallic acid	500	0.44	500	1.23
Ferulic acid	275	1.55	275	2.21
Quercetin	500	6.65	500	3.77
Rutin	500	4.67	500	1.98

(Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5µm and dimensions (150 × 4.6 mm). A binary gradient elution system composed of 0.1% formic acid in acetonitrile as solvent A and 0.1% formic acid in HPLC grade water as solvent B. (Adamu et al., 2018). The gradient elution is as follows: 0-10 min, 18-20% A; 10–25 min, 20-30% A; 25-45 min, 30-70% A; 45-46 min, 70-80% A; 46–50 min, 80% A. The mobile phase flow rate was 0.8 ml/min, and column temperature was maintained at 40 ◦C. The DAD detector was set at 254 nm.

Validation of HPLC method

The HPLC method was validated in terms of linearity and precision according to ICH guidelines (ICH Topic Q2B, 1996). The calibration curve was constructed by running several mixed standards of different concentrations in triplicate. The correlation coefficient was determined using a linear regression model. The precisions and reproducibility of HPLC peak are measurements of quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid were calculated as the relative standard deviations (RSDs) of six repeated runs and six replicate runs.

RESULTS AND DISCUSSION

UPLC method validation of quantitative analysis

Linearity and detection limit

The linearity for six phenolics was established by plotting the peak area (Y) versus concentration (x) of each which was expressed by the equation given in Table 1. The linearity of all the calibration curves showed good linear regression coefficients between ($r2 = 0.996$-0.999) within test ranges.

Precision and reproducibility

Method precision and reproducibility were evaluated by the analysis of six injections of the same sample solution and injection of six replicates of the same sample, respectively. The percentage relative standard deviations (%RSDs) of peak area (PA) of characteristic peaks in the precision test were found in the range of 0.44-6.65%, whereas in the reproducibility test the RSDs of PA were also between 0.77 and 3.77%, respectively. All results indicated that the method of HPLC fingerprint analysis was valid and satisfactory.

As shown in Table 2, the precisions and reproducibility based on peak area and retention time measurements of six components were found to be satisfactory for all the target phenolics. All these illustrated that UPLC-DAD method was precise, and sensitive enough for simultaneous quantitative determination of quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid. The entity and content of bioactive components of medicinal plants are always highly variable. Considering the variety of the components in the seeds of kola nut (*C. nitida*), bitter kola (*G. cola*) and wonderful kola *(B. coriacea*) and in order to give the most chemical information of these plants, HPLC provide a suitable technique which can perform both qualitative and quantitative analysis of the bioactive compounds in these
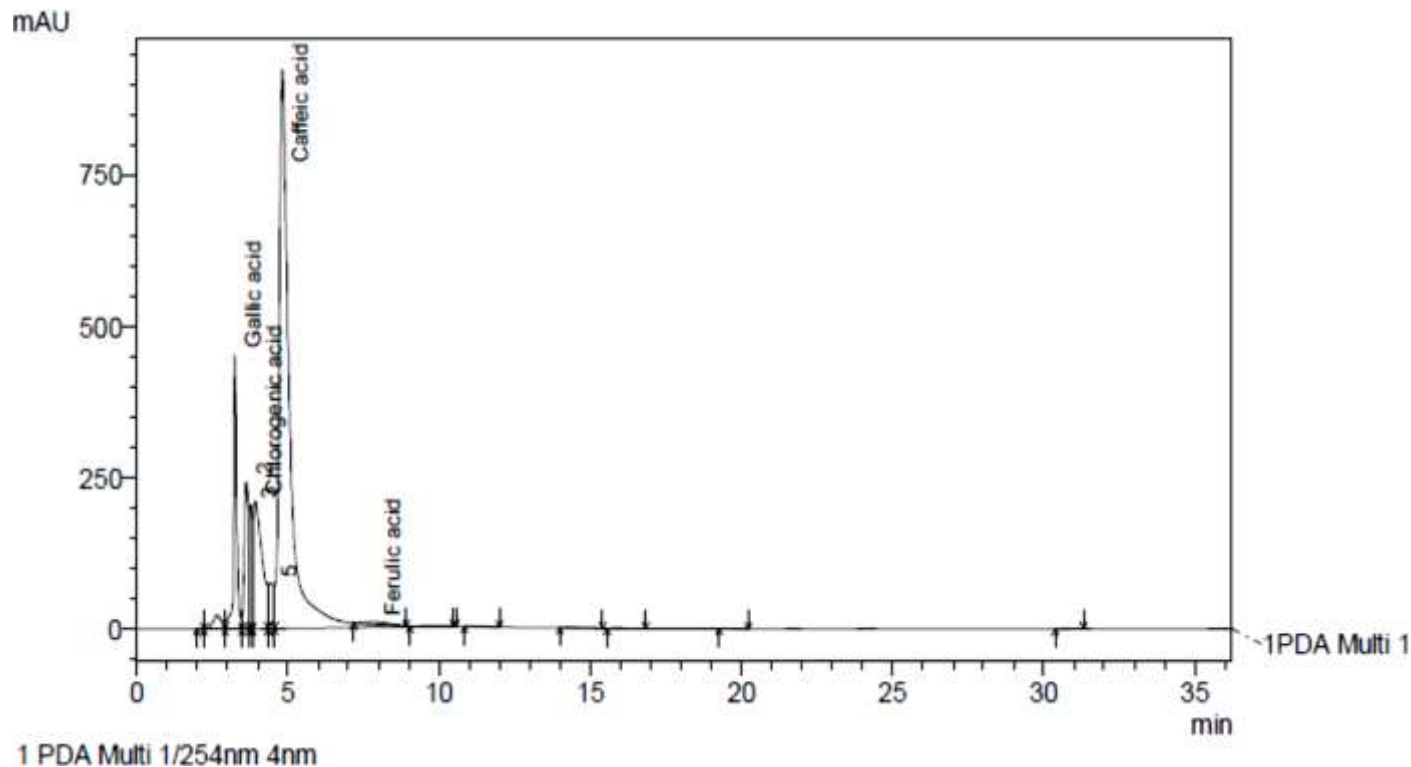
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Figure 1. HPLC chromatogram of *C. nitida*.

plants. The HPLC profile of all the components in the seeds of kola nut (C. *nitida*), bitter kola (*G. cola*) and wonderful kola *(B. coriacea)* was established. Bioactive compounds which are previously reported to shows good antioxidant properties including: quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid were quantify in each of the samples.

To provide information of the bioactive compounds in these kolas and for best separation in the chromatograms, the mobile phase and its flow rate, conditions for elution, column temperature and detection wavelength were investigated in this study. Variation in the ratio of water to acetonitrile in the mobile phase provided improvement in separation. In a previous study, Xu et al. (2009), observed that acetonitrile/water mobile phase system achieved better resolutions for phenolic acids. In addition the retention behavior of phenolic acids on the reversed-phase HPLC column was significantly affected by the pH of the mobile phase. So the addition of 0.1% formic acid into mobile phases helped to achieve a good baseline and satisfactory resolution of the phenolics. The detection wavelength was set at 254 nm because most of the characteristic components have satisfactory sensitivity at this UV wavelength. The HPLC conditions developed in this study produced insight into the fingerprint pattern of the different kolas. The chromatographic peaks were identified by comparing their retention time and UV spectrum with that of each reference compound, which was eluted in parallel with a series of mobile phases.

Quantitative analysis of the six phenolics in the samples

Quercetin, caffeic acid, gallic acid, ferulic acid, rutin and chlorogenic acid are known phytochemical constituents proven to be good natural antioxidants. It could be seen from Figures 1 to 3 that the investigated phenolics and other compounds in these kolas were separated using the developed UPLC-DAD method. A total of 7, 12 and 15 characteristic peaks were identified in the HPLC profile of *C. nitida*, *G. cola* and *B. coriacea* respectively. Six of the characteristic peaks were assigned by comparing the UV spectra and their retention time with those of the reference compounds. In *C. nitida* peak 1, 4, 6, and 7 were identify as gallic acid, chlorogenic acid, caffeic acid and ferulic acid. Peaks 1, 5, 7, 8 and 10 were identified as gallic acid, caffeic acid, rutin, ferulic acid and quercetin in *G. cola* and for *B. coriacea* chromatogram (Figure 3) peak 2, 4, 5, 7 and 9 were identify as gallic acid, chlorogenic acid, caffeic acid, rutin and ferulic acid respectively.

The quantitative analysis data (Table 3) showed that the quantity of these compounds in *C. nitida* is caffeic acid (101.24 mg/g), chlorogenic acid (36.35 mg/g), gallic
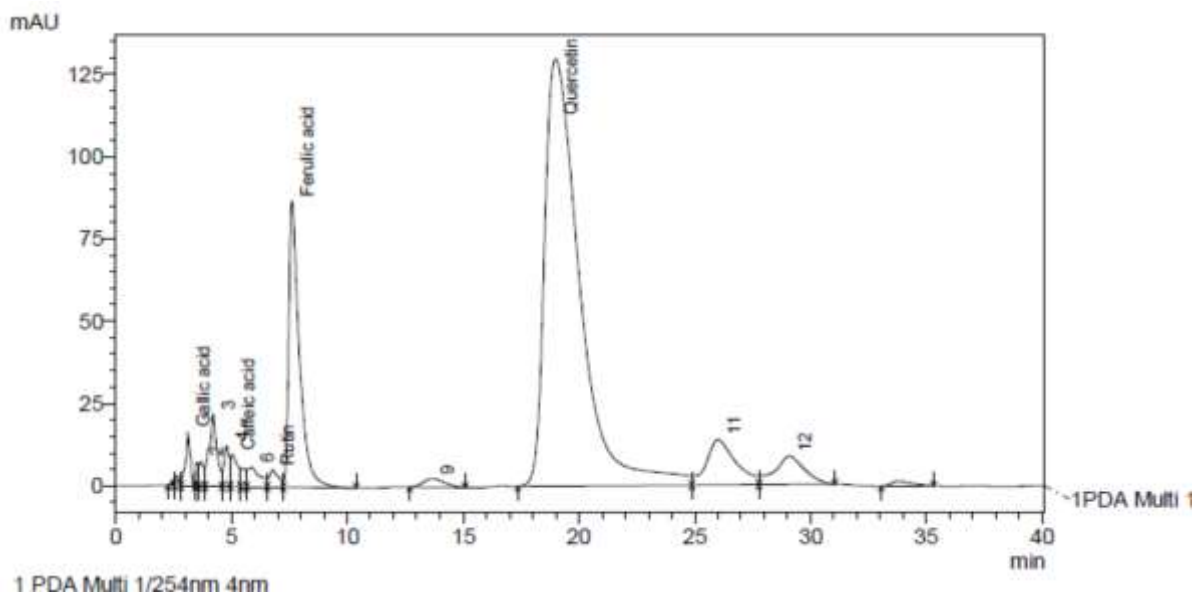
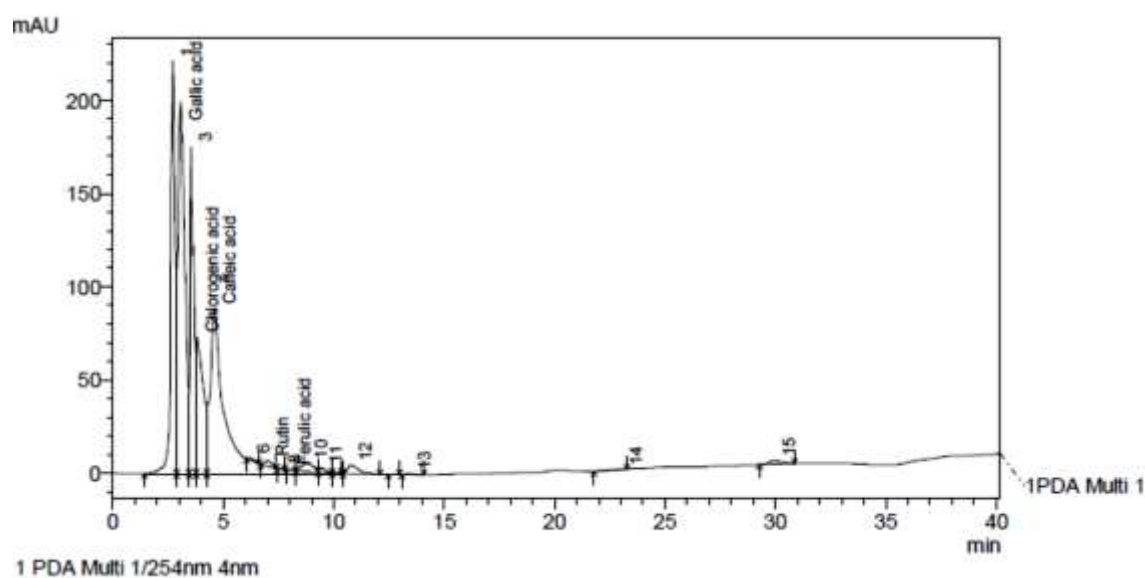**Figure 2.** HPLC chromatogram of *G. kola.*

Figure 3. HPLC chromatogram of *B. coriacea.*

acid (16.99 mg/g) ferulic acid (1.47 mg/g) while quercetin and rutin were not detected. In *G.kola* caffeic acid is (0.84 mg/g), gallic acid (1.02 mg/g), ferulic acid (21.83 mg/g), quercetin (53.24 mg/g), rutin (0.49 mg/g) and chlorogenic acid was not detected. *B. coriaceae* has caffeic acid (1.03 mg/g), chlorogenic acid (0.33 mg/g), gallic acid (1.07 mg/g) while ferulic acid, quercetin and rutin were not detected.

Table 3 also showed that the most abundant compound in the three species of kolas is caffeic acid in *C. nitida* follow by quercetin in *G. kola.* Chlorogenic acid was equally as abundant as caffeic acid in *C. nitida* but very minute in *B. coriacea* and absent in *G. kola.* This shows that the levels of these compounds present in individual samples varied considerably and the concentration of each compound in different samples was significantly different. Although *C. nitida* is the most widely and regularly consumed, because of its varied socio-cultural

Table 3. The compounds in the three kola species (mg/ml).

Compound	*C. nitida*	*G. kola*	*B. coriacea*
Caffeic acid	101.24	0.84	1.03
Chlorogenic acid	101.24	ND	0.33
Gallic acid	16.99	1.02	1.07
Ferulic acid	1.47	21.83	ND
Quercetin	ND	53.24	ND
Rutin	ND	0.49	ND

ND= not detected.

importance. This study has also shown that *C. nitida* contains high amounts of phenolics compounds as compare to the other species of kola seeds investigated in the study.

The seeds of these kolas have received great interest due to their social significance and pharmacological properties. Phenolic compounds in synergy with the other compounds investigated in this study are responsible for their health benefits. Studies have shown that caffeic acid possesses anti- inflammatory, anti-mutagenic, anti-bacterial and anti-carcinogenic properties (Genaro-Mattos et al., 2015). Chlorogenic acid is an ester of caffeic acid have antioxidant properties which are propose to play a crucial role in protecting food, cell and many organs from oxidative degenerative. It has been discovered from research that diet that contain CGA compounds plays a vital role in preventing various diseases and ailments associated with oxidative stress such as cancer, cardiovascular, aging, diabetes, neurodegenerative diseases, anti-bacterial and anti-inflammatory activities (Ayelign and Sabally, 2013). Gallic acid has been proved to have potential preventive and therapeutic effects in many diseases, where the oxidative stress has been implicated including cardiovascular diseases, cancer, neurogenerative disorders and in aging.

Gallic acid has been reported to possess good pharmacological activities and could be exploited lead compound for new drug development (Nayeem et al., 2016). Ferulic acid is an antioxidant found in many staple foods, such as fruits, vegetables, cereals, coffee and in many other plants, exhibiting a wide range of pharmacological activities such as antioxidant, neuroprotective, anticancer, anti-diabetic, cardio-protective, anti-inflammatory and other activities (Gohil et al., 2012). Quercetin is present in most plants and is known for its biological activities such as antioxidant, antiviral, anticancer, antimicrobial, and anti-inflammatory and others (Maalik et al., 2014*)*. Rutin is a flavonol, richly found in plants, such as passion flower, buckwheat, tea, and apple. It has demonstrated a number of pharmacological activities which includes: antioxidant, vaso-protective, anti carcinogenic, neuroprotective and

cardio protective (Ganeshupurkar and Saluje, 2017). The result from the study suggests that *C. nitida* with high phenolic acid contents could exhibit a better antioxidant activity than *G. kola* and *B. coriacea*.

Conclusion

HPLC method shows peaks with reasonable heights and fair resolutions were assigned as ''characteristic peaks''for identification of these kolas. Using this analytical method, some phenolics and flavonoids compounds were quantified. It can be concluded from the results that all the three species of kola contain phenolics compounds but only *Garcinia kola* contain the two flavonoids compounds (quercetin and rutin) investigated in this study. The most abundant compound in the three species of kola is caffeic acid in *C. nitida* follow by quercetin in *G. kola*. *C. nitida* is the most widely and regularly consumed, because of its varied socio-cultural importance. This study has also shown that it contains high amounts of phenolics compounds compared to the other two species of kola investigated in the study. The method described offers a good quantitative analytical approach to phenolic acids determination in plant extracts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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