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Review

Propolis as natural additive: A systematic review

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Propolis extract is a resin collected by the bees and contains numerous active substances with a recognized pharmaceutical potential use. The aim of this study was to conduct a systematic review of studies with the use of propolis as a food additive and the microencapsulation technology for incorporation of the product in food. Studies in Portuguese, English and Spanish were identified from online databases, Lilacs, Pubmed, Medline, Science Direct, Google Scholar/Google Academic, Bioline International, Springer Link, Vadlo and HighBeam Research. Studies published between 1979 and May 2018 that assessed the use of propolis as a natural food additive and microencapsulation technologies were included. The analysis showed that there are few studies about the use of propolis as a natural additive in food for human consumption, in spite of several authors bringing the importance of bioactive compounds of propolis. Based on this review, further studies are needed to cover the incorporation of propolis in food, considering its potential as a natural additive and its antioxidant and antimicrobial properties. It also shows the need for studies to enhance the technology of microencapsulation in order to preserve the characteristics of interest and mask those that may influence the foods acceptance.

Key words: Additives, bioactive principles, food safety, propolis, microencapsulation.

INTRODUCTION

The concern for safety in the use of synthetic preservatives in foods and the preference for natural products have led to the increase in research on antioxidants and other preservatives derived from natural sources such as cocoa, rice, apple, red onion, oregano, rosemary, honey and propolis (Jiang et al., 2013). In this context, apiculture products (propolis) have been highlighted for use in foods either for therapeutic

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purposes, or to be used as food, and their use is one of the oldest universal practices. Propolis is a resinous honeybee product and a traditional therapeutic agent in folk medicine. It is produced by bees (*Apis mellifera* L.) to meet some needs in the hive, such as filling in gaps, reducing input and output of the hive openings, mummification of insect bodies, covering the inner walls of the hive and the inside cells, repairing damaged honeycombs and consolidation of mobile honeycombs (Falcão et al., 2010; Moreira et al., 2011; Bankova et al., 2016; Cuesta-Rubio et al., 2017; Frozza et al., 2017; Thamnpoulos et al., 2018).

The propolis antimicrobial activity was already empirically known by the ancient Egyptian priests that used it in the embalming process to protect the mummies from fungi and bacteria attack (Gonsales et al., 2006). This antimicrobial property is mainly attributed to the presence of flavonoids with phenolic acids and esters, phenolic aldehydes and ketones (Pietta et al., 2002). Related to the antibacterial activity, the mechanism is considered complex and can be attributed to the synergism among flavonoids, hydroxy and sesquiterpenes (Krol et al., 1993). The product has a complex chemical composition, with over 300 compounds and minerals such as aluminum, calcium, strontium, iron, copper, manganese and minor amounts of vitamins B1, B2, B6, C and E (Pietta, 2002; Khalil et al., 2015). It stands out for its antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, hypotensive, healing and anesthetic properties (Burdock, 1998). Propolis is known to be the most concentrated source of bioflavonoids, which are the main antioxidants found in plant extracts. The activity of phenolics and other bioactive compounds in inhibiting autoxidation in some foods and biological systems has been attributed to their redox properties (Ukulo et al., 2013).

In its native application, the primary function of propolis is biocidal action against bacteria and invasive fungi, suggesting its potential for industrial application. In the foods application, propolis has been used in industry as a preservative in meat products, increasing the shelf life of frozen meat and other foods (Netikovã et al., 2013; Feás et al., 2014; Bankova et al., 2016, Vargas-Sánchez et al., 2014; Casquete et al., 2016). The action mechanism is still not fully understood due to the existing synergy between the different substances in propolis and the presence of multiple targets in each affected organism (Seidel et al., 2008). The chemical properties of the propolis are highly variable and depend on the source plant and the flora at the site of collection. These different chemical compositions do not always lead to significant differences in physicochemical and pharmacological activities. Nevertheless, some difference between the diterpene-rich Mediterranean propolis that has weak antioxidant activity and the poly-phenol-rich types (mainly Brazilian green and poplar type) has been verified, while its antibacterial properties are comparable to those of poplar type (Bankova et al., 2016). Mechanisms to enrich food products with beneficial bioactive compounds have been developed by the food industry. To ensure minimal impact on the organoleptic and qualitative properties of developed products, the use of encapsulation technology is a powerful tool, since it enables the protection of a wide range of compounds by their embedding into a protective matrix (Dordevic et al., 2014). The use of this technology, since the 1950s, has increased in food industry, because the encapsulated material can protect food from heat, moisture, oxidation, or other chemical reactions in extreme conditions, increasing its stability and maintaining its viability (Gouin, 2004). Based on these considerations, this study aimed to conduct a systematic review of the use of propolis as a food additive and the microencapsulation technology for incorporation of the product in food.

MATERIALS AND METHODS

Search strategy

The data were selected by searching the online databases: Latin American and Caribbean Health Sciences (LILACS), PubMed, Medical Literature Analysis (MEDLINE), Science Direct, Google Scholar/Google Academic, Bioline International, Springer Link, Vadlo and HighBeam Research. The selection criteria considered the articles published in English, Portuguese and Spanish, available from 1996 to 2018. The exclusion methodology was for abstracts and book chapters. The key words used in Portuguese were: *"própolis", "própolis como aditivo natural", "própolis em alimentos", "microencapsulação", "microencapsulação em alimentos", "microencapsulação da própolis*" and *"microencapsulação da própolis em alimentos*"; in English: "propolis", "propolis as natural additive", "propolis in foods", "microencapsulation", "food microencapsulation", "Microencapsulation of propolis" and "Propolis microencapsulation in food"; in Spanish: *"propóleos", "propóleo como un aditivo natural", "propóleos en los alimentos", "microencapsulación", "microencapsulación en los alimentos", "microencapsulación de propóleo", and "microencapsulación de propóleo en los alimentos*".

The analysis of the studies was carried out using the summarization of the information in articles, such as: author, year of publication, sample, methodology used, and main results found.

RESULTS AND DISCUSSION

Incorporation of propolis in foods

Research in the mainly databases inserting the term "propolis" identified a total of 18.536 articles (Figure 1). Based on the total of identified articles, the importance of propolis to the scientific community in the fields of microbiology, chemical and pharmaceutical areas can be measured. However, when entering in the databases the subject "Propolis as a natural food additive" 16 articles were viewed and in the search for "Propolis in foods" 12 articles were identified (Figure 2). Other databases

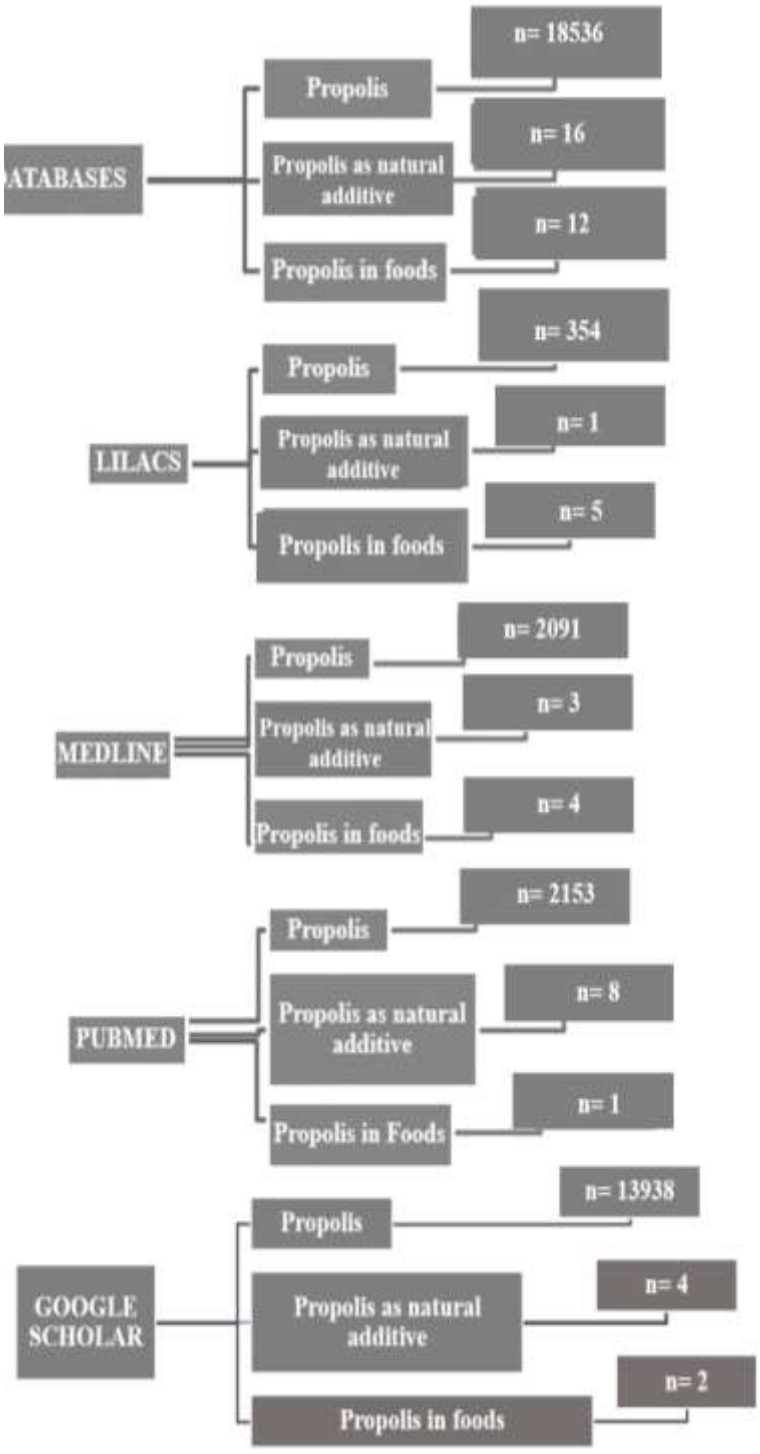

Figure 1. Records identified through data bases searching Medline, Pubmed, Lilacs and Google Scholar related to keywords: “Propolis”, “Propolis as a natural additive” and “Propolis in foods”.

presented the following results: Science Direct - Propolis: 1.074 matching articles; Bioline International-propolis: 31 matching articles; Springer Link-propolis: 855 matching articles; Vadlo-propolis: about 2.480.000 matching articles; and HighBeam Research-propolis: 1.074 matching articles.

The analysis showed that there are few reports in the literature on propolis as a natural additive in food for

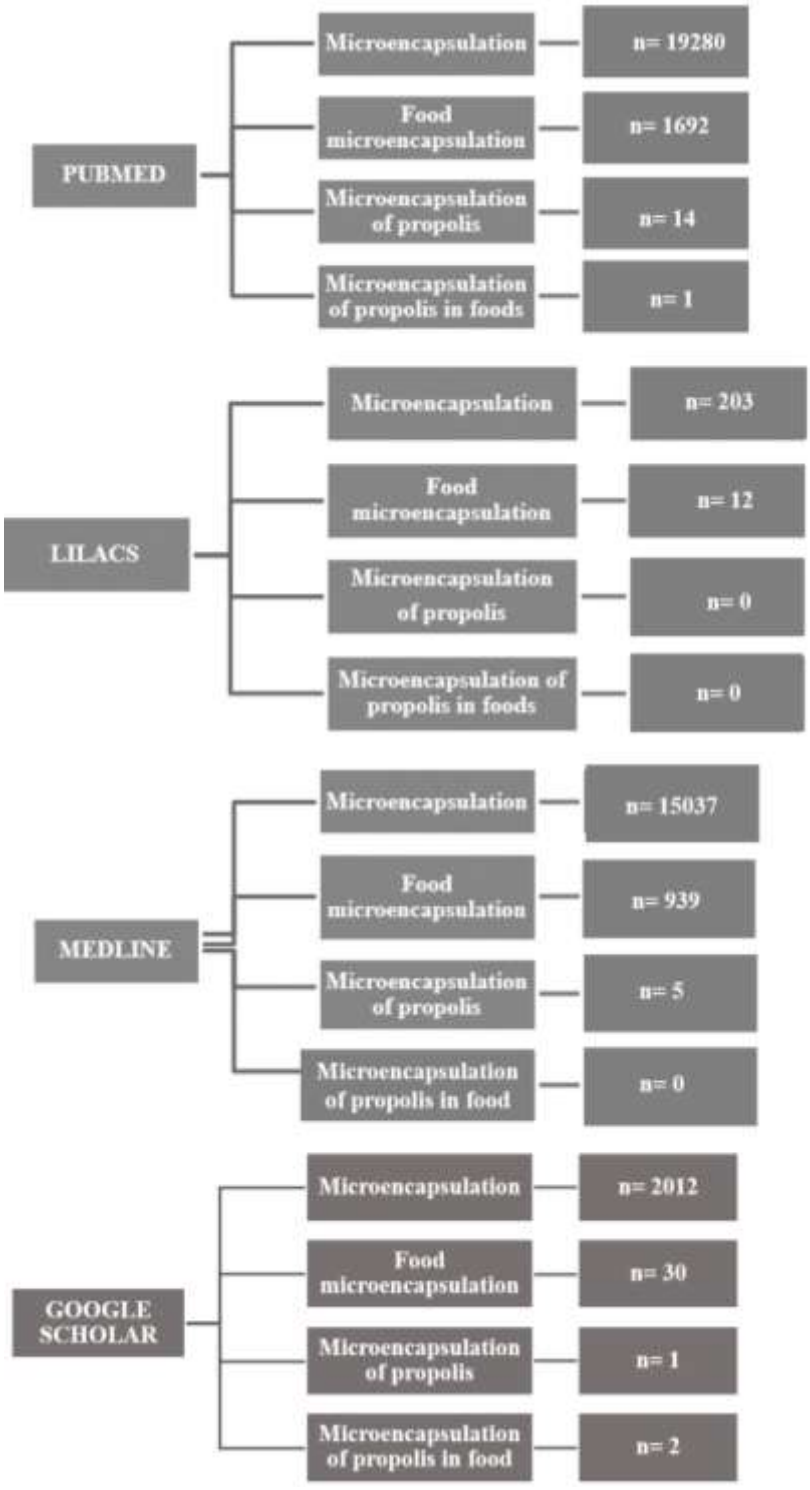

Figure 2. Records identified through data bases searching: Medline, Pubmed, Lilacs and Google Scholar related to keyword “microencapsulation”, “food microencapsulation", “microencapsulation of propolis” and “propolis microencapsulation in food”.

human consumption and propolis in foods. Regarding the use of propolis as antioxidants natural source, a study performed by Mendiola et al. (2010) highlights the use of natural sources of antioxidants to replace synthetic

Table 1. Biological properties of propolis.

Propolis properties	Studies	References
Antioxidant	Dihydroxychalcones eliminates riboflavin (Rf) – photogenerated reactive oxygen species (ROS).	González et al. (2015)
	Elimination of free radicals, inhibiting lipid peroxidation and hemolysis of human erythrocytes.	Coelho et al. (2015)
	Sinapic acid and rutin potential alternative as a natural antioxidant.	Espinosa et al. (2015)
	Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal	Moreira et al. (2008)
Anti-inflammatory	Hydroalcoholic extract of propolis (HPE) reduces HSV-2 in vaginal tissues of animals	Sartori et al. (2012)
	Anti-inflammatory and immunomodulating activity of caffeic acid phenethyl ester (CAPE).	Armutcu et al. (2015)
	Inhibition of hyaluronidase enzyme.	Coelho et al. (2015)
Antifungal	Activity against *Candida albicans and Candida glabrata*	Boisard et al. (2015)
Antibacterial	Inhibitory effect of the polyphenols on the growth of *Streptococcus mutans*	Veloz et al. (2015)
	Activity against *Staphylococcus aureus*	Boisard et al. (2015)
	Activity against *Listeria monocytogenes*	Thamnpoulos et al. (2018)
	Antimicrobial activity, phenolic profile and role in the inflammation of propolis	Silva et al. (2012)
Antiviral	Activity of propolis extract against feline calicivirus, canine adenovirus 2 and bovine viral diarrhea.	Cueto et al. (2011)
	Activity of C-glycosyl flavones present in geopropolis against anti-herpes simplex virus (HSV).	Coelho et al. (2015)
	Parvovirus swine inhibition (PPV) *in vitro* and *in vivo*	Ma et al. (2015)

antioxidants using plants, fruits, Spirulina, tubers and propolis. The authors demonstrate that propolis has a significant amount of antioxidants, indicating that they can be an alternative replacement for synthetic antioxidant (Table 1). The antimicrobial and antioxidant properties attributed to propolis are valuable for the food industry due to its effect in delaying lipid oxidation and improving the food shelf life. These characteristics are due to the presence of chemical compounds like flavonoid, pinocembrin, flavonol, galagina and caffeic acid phenylethyl ester, which exhibit-based-action mechanism probably in inhibiting bacterial RNA polymerase (Funari and Ferro, 2006).

Other components such as flavonoids, caffeic acid, benzoic acid and cinnamic acid, probably act at the level of membrane or cell wall of the microorganisms, causing structural and functional damage (Funari and Ferro, 2006). Moreover, polyphenols contain an extensive range of other compounds with the ability to remove excess free radicals from our body (Santos, 2015). Table 2 shows studies related to use of propolis as a natural additive in foods. Some of these studies are the basis for future work in order to add propolis to food for human consumption, because it contains biological properties necessary for inhibition of microbial growth and consequently for food preservation.

It is important to consider that the consumption of foods containing chemicals that are not nutrients, can lead to the appearance of different effects in different degrees and with several designations such as: toxic effect, harmful, detrimental adverse or unexpected (Menezes, 2005), associated with the emergence of allergies, behavioral changes such as hyperactivity and cancer (Moutinho et al., 2007). Such prospects will obligate the food industry to eliminate the use of chemical preservatives and adoption of natural alternatives to food. In recent times, the increasing interest in the food industry to find natural additives have stimulate the efforts both in obtaining bioactive compounds in natural raw materials, and in development of stable products and functional derivatives (Silva et al., 2013).

Valero et al. (2014) evaluated the effect of natural additives as propolis or essential oils on meat quality of crossbred (Aberdeen Angus vs. Nellore) bulls. Addition of natural additives as propolis extract or cashew and castor oils in the diet of bulls when they are finished in a feedlot

Table 2. Studies related to use of propolis as a natural additive in foods.

References	Countries	Title	Description
Koc et al. (2007)	Turkey	Antifungal activity of propolis in four different fruit juices.	This study evaluated the effect of ethanol extract of Turkish propolis treatments in juices nonpasteurized fruit (apple, orange, white grape and mandarin) against six different yeasts. The authors indicated the potential use of propolis as an alternative to chemical fruits juices preservation agents.
Özdemir et al. (2009)	Turkey	The effects of ethanol-dissolved propolis on the storage of grapefruit cv. Star Ruby	Effect of propolis on the storage life of Star Ruby grapefruit was investigated. Fruits were dipped in ethanol-extracted propolis (1%, 5% and 10%). This study showed that the weight loss was significantly higher in fruits without treatment. The best concentration of propolis for a period of 5 months was 5%, demonstrating that the propolis has a strong anti-microbial effect and limits the growth of microorganisms.
Kim et al. (2013)	Korea	Synergistic effect of propolis and heat treatment leading to increased injury to *Escherichia coli* O157:H7 in ground pork.	This study determined the thermal inactivation of *Escherichia coli* O157: H7 in the presence of propolis in culture and ground pork. According to the authors, *E. coli* O157:H7 was inhibited in culture and in ground meat by decreasing the heat resistance, demonstrating a synergistic effect.
Kamel et al. (2015)	Egypt	The effect of propolis and sodium metabisulfite as postharvest treatments on pomegranate arils storage.	This study compared the effect of sodium metabisulfite, extract of propolis and mixed solution for treatment of aryls during storage under refrigeration. Highest effect at 25 days was achieved when combined treatment was used. The overall result showed that both sodium as propolis extract has the potential to maintain arils attributes. However, the use of higher concentration of propolis as a single treatment demonstrated negative effects.
Thamnpoulos et al. (2018)	Greece	Inhibitory activity of propolis against *Listeria monocytogenes* in milk stored under refrigeration	The objective of this study was to develop a protocol for adding propolis into milk and to determine whether the addition of propolis can confer anti-listerial activity during the storage of milk under optimal or improper refrigeration conditions. Results highlight the strong anti-listerial potential of propolis in milk.

did not change meat qualities. The works related to potential of the propolis as biological and chemical-pharmaceutical product highlighting the need for research on the potential of propolis as a natural additive in food for human consumption, with emphasis on how the compounds found in propolis can replace chemical additives commonly used in foods. Some research confirms that low concentrations of propolis extract can be used as antimicrobial and antioxidant substances for food protection (Mendiola et al., 2010; Bernardi et al., 2013).

However, propolis toxicological studies are required, considering the cumulative effects or synergistic protection. It is important not only to know the specific properties that convert the food additive, but also all actions and contra-indications, especially those derived from prolonged use (Salinas, 2002). Araújo et al. (2011) observed little toxicity of a hydroalcoholic extract of

Brazilian propolis. Similar results were reported by Mohammadzadeh et al. (2007), who found no toxic effects after the ingestion of Iranian propolis. The application of propolis in food, however, has limitations due to the strong flavor and aroma and its difficult solubility. Because of this, usually propolis is administered in alcoholic solutions, which limits its application in food. These disadvantages result in storage problems, transport, development and management (Silva et al., 2013).

Microencapsulation technology

Related to microencapsulation technology, a total of 36.532 articles was identified in the mainly databases, using the term “Microencapsulation” (Figure 2). Other databases presented the following results: Science Direct-microencapsulation: 824 matching articles; microencapsulation of food: 345 matching articles; Springer Link - propolis in microencapsulation: 13 matching articles; Vadlo - microencapsulation: about 201.000 matching articles; and High Beam Research - microencapsulation: 824 matching articles; microencapsulation of food: 345 matching articles.

Microencapsulation is the packaging of solid, liquid droplets or gaseous material with fine polymer coverage. This procedure involves the incorporation of food ingredients, enzymes, cells or other materials in small caps. It is a barrier to prevent chemical reactions and allow the modified release of ingredients under specific conditions and speed, masking odors and tastes (Rosenberg et al., 1990; Gouin 2004, Đordevic et al., 2014). The encapsulated material can be protected in extreme conditions, increasing its stability and maintaining its viability (Gouin, 2004).

By filtering the search for “food microencapsulation”, 2.673 articles were found, demonstrating a widespread technique. However, research on the use of the term “Microencapsulation of propolis” are still limited, having identified only 19 articles, while for the search for the term “Propolis microencapsulation in food” two articles was found. Therefore, it can be concluded that the studies about the potential use of propolis as a food additive is very small in the scientific literature, as shown in Table 3.

Favaro-Trindade et al. (2008) evaluated the effects of microencapsulation of the ethanol extract of propolis on solubility in water and the product properties *in vitro* to release components. To this end, encapsulation using ciclodextrins was tested and samples of propolis from Southern Greece. The authors observed increased water solubility of various bioactive components of propolis extracts and demonstrated that the net release of the compounds depends not only on the chemical properties of the product, but also on the relative abundance in the sample of propolis extract.

Work performed by Koo et al. (2002) discusses the use of spray drying method for preparing microparticles of propolis, using gelatin as the polymer. It was shown that this method besides being cheap and practical, maintains the activity of propolis extract against *Staphylococcus aureus*. This study represents new perspectives for microencapsulation technique of propolis. Studies related to the use of propolis as a food additive was developed by Gomes et al. (2011) (Table 3). The authors evaluated the antimicrobial efficacy of extracts of natural compounds against Gram-positive and Gram-negative bacteria, the antimicrobial activity of microencapsulation of natural compounds beta-cyclodextrins and the degree of radiosensitization of *Salmonella* spp. on spinach sprayed with microencapsulated compounds. Data consisted the effectiveness of all compounds in inhibiting the growth of Gram-positive and Gram-negative; both of them were used directly as extract and microencapsulated. Still, according to the authors, the microencapsulation improved stability, solubility and masked the strong odor and off flavor. The combination of irradiation dosage levels <1 kGy and spray microencapsulated natural antimicrobial compounds ensured a reduction of 5 log cycles in the population of *Salmonella* spp., without detrimental effects on product quality.

According to Bernardi et al. (2013), propolis prevented lipid oxidation in salami during storage, but showed lower sensorial acceptance. To improve sensorial acceptance of fish burgers, Spinelli et al. (2014) used ingredients such as potato flakes and extra virgin olive oil and obtained a final fish product with good acceptability. Burgers showed increase of both phenolic content and antioxidant activity. Reis et al. (2017) evaluated the characteristics of the microencapsulated propolis co-product extract (MPC) and their effects on stability and sensory quality in burger meat. The efficiency of the microencapsulation technology regarding the phenolic compounds content was high. Smell and flavor in burger meat containing MPC showed lower grades than the ideal scale. However, color, appearance, and texture demonstrated ideal grades (Table 3).

CONCLUSION AND FUTURE PERSPECTIVE

Based on the systematic review, it was verified that the scientific articles in the period studied, focused mainly on studies to investigate biological and chemical - pharmaceutical potential, with only a few studies about the incorporation of propolis in food. From this perspective, considering the potential of propolis as a natural additive and its antioxidant and antimicrobial properties, there are necessary further studies covering the incorporation of propolis in food, considering its potential as a natural additive and its antioxidant and antimicrobial properties. It also shows the need for

Table 3. Studies using the microencapsulation technology for food.

References	Title	Description
Gomes et al. (2011)	Microencapsulated antimicrobial compounds as a means to enhance electron beam irradiation treatment for inactivation of pathogens on fresh spinach leaves.	The study aimed to evaluate the effectiveness of the use of natural antimicrobials. The minimum inhibitory concentration of 5 compounds and natural extracts (trans cinnamaldehyde, eugenol, garlic extract, propolis extract, and lysozyme with EDTA were determined against *Salmonella* spp. and *Listeria* spp. The efficacy of the microencapsulated compounds was tested by spraying onto the surface of contaminated spinach. The results confirmed that the combination of microencapsulated spraying with electron beam irradiation is effective in enhancing the lethal effects of irradiation.
Nori et al. (2011)	Microencapsulation of propolis extract by complex coacervation	This study aimed to obtain encapsulate propolis extract by complex coacervation using isolated soy protein and pectin as encapsulant agents. Encapsulate propolis extract was obtained in the form of powder, alcohol-free, stable, with antioxidant property, antimicrobial activity against *Staphylococcus aureus* and with the possibility of controlled release in foods.
Spinelli et al. (2014)	Microencapsulated propolis to enhance the antioxidant properties of fresh fish burgers	The study aimed to evaluate the antioxidant properties of microencapsulated propolis added in fish burgers. To improve sensorial acceptance, ingredients such as potato flakes and extra virgin olive oil were tested and optimized to give a final product with good acceptability. Burgers showed increase of phenolic content and antioxidant activity.
Koga et al. (2016)	Consumer acceptance of bars and gummies with unencapsulated and encapsulated resveratrol.	The objective of this research was to show the application of resveratrol in microcapsules in food to be consumed. The microencapsulation in a matrix of sodium caseinate was used as a strategy to overcome the bitterness of resveratrol. The microcapsules resveratrol there was a significant lower overall taste than the control with the same protein and / or content of resveratrol.
Rutz et al. (2016)	Elaboration of microparticles of carotenoids from natural and synthetic sources for applications in food.	The study aimed to encapsulate the palm oil and β -carotene with chitosan tripolyphosphate/chitosan or sodium/carboxymethylcellulose and evaluate the performance of these microparticles in food systems by analyzing their release profile under gastric conditions and simulated intestinal. The encapsulation efficiency was greater than 95%.
Rached et al. (2016)	*Ceratonia siliqua* L. hydroethanolic extract obtained through ultrasonication: antioxidant activity, phenolic compounds profile and effects in yogurts functionalized with their free and microencapsulated forms.	Bioactive extracts were obtained from carob pulp powder through an ultrasonic extraction process and then evaluated in terms of antioxidant activity in yogurt. The study confirmed the efficiency microencapsulation to stabilize the functional ingredients in food matrices almost maintaining the structural integrity of polyphenols extracted from carob pulp and improving in addition, the antioxidant potency of the final product.
	Physico-chemical characteristics of microencapsulated propolis co-product extract and its effect on storage stability of burger meat during storage at - 15°C.	Characteristics of the microencapsulated propolis co-product extract (MPC) and their effects on stability and sensory quality was evaluated in burger meat. The efficiency of the microencapsulation technology regarding the phenolic compounds content, was high. Smell and flavor in burger meat containing MPC showed lower grades than the ideal scale. However, color, appearance, and texture demonstrated ideal grades.

studies to enhance the technology of product micro-encapsulation in order to preserve the characteristics of interest and mask those that may influence the food acceptance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Anti-diabetic and some haematological effects of aqueous and ethanol leaf extract of *Eriosema psoraleoides* in alloxan-induced diabetic Wistar rats

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This study investigates the anti-diabetic and some hematological effects of aqueous and ethanol leaf extracts of *Eriosema psoraleoides* in alloxan-induced diabetic Wistar rats. Forty-eight albino mice were used for the lethal dose (LD_{50}) study, while twenty-eight Wistar rats were used for the diabetic and haematological study. The result of the phytochemical analysis shows some important phytochemicals that could confer some health benefits. The extracts had lethal doses (LD_{50}) of 4000 and 5000 mg/kg^{-1} body weight, which indicate that the extract was safe to a greater extent. The baseline blood glucose concentration presents non-diabetic glucose level 86.25±8.26 mg dL^{-1}. Diabetes was induced with alloxan monohydrate. A significant decrease ($p<0.05$) in blood glucose levels in the test animals were observed when compared with positive control. The extracts had a significant effect on blood glucose and haematological levels of the treated rats closer to that of the standard drug (glibenclamide) with significant ($p<0.05$) increase in red blood cell (RBC) and significant ($p<0.05$) decrease in neutrophils and lymphocytes in all test groups relative to positive control. However, white blood cell (WBC) showed significant decrease ($p<0.05$) in groups which received aqueous extracts when compared with positive control. The results of the study showed that the extracts of *E. psoraleoides* leaves exhibited significant anti-diabetic activities against alloxan-induced diabetes Wistar rats. It suggests the extracts possess bioactive compounds that when properly harnessed could help in improving the health state of diabetic patients.

Key words: *Eriosema psoraleoides*, anti-diabetic, alloxan induced diabetic rats, haematological parameters.

INTRODUCTION

Diabetes mellitus has become a major public health concern, especially in the developing countries. It is characterized by absolute or relative deficiencies in insulin secretion, action or both associated with chronic

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hyperglycemia and disturbances in carbohydrate, lipid, and protein metabolism (Rahmati et al., 2013). It is characterized by chronic high blood glucose that could lead to morbidity and mortality (Mohammed et al., 2007). There is an alarming rate of diabetes cases worldwide. It is predicated that about 366 million people are likely to be diabetic by the year 2030 (Wild et al., 2004). This is because none of the antidiabetic drugs could give a long term glycaemic control without causing any adverse side effects (Singh et al., 2007). Meanwhile, medicinal plants that are effective in controlling plasma glucose level with minimal side effects are commonly used in under developed countries as alternative therapy. In Africa, hundreds of plants are used traditionally for the management and/or control of diabetes mellitus. Unfortunately, only a few of such medicinal plants have been scientifically validated (Tanko et al., 2007). One of the plants commonly used in *Eriosema psoraleoides* ((Lam.) G. Don) is a tropical plant belonging to the family Leguminosae. It is an erect herb or sub-woody shrub that can grow 1 to 2.5 m high from a perennial woody root-stock. It is widely distributed throughout Tropical Africa and South Africa (Hyde and Wursten, 2010). In Nigeria, the locals of Nsukka call it *ENYI AGBAOKWU AGBUGBA*, in Senegal it is *MANDING-BAMARA* nomko blé (JB) and in Sierra Leone they are *KORANKO* kouŜe (NWT) and yaŋgune (NWT) *LOKO.* The fruits, leaves and roots are mostly used traditionally for the treatment of various diseases and infections such as cutaneous and subcutaneous parasitic infections, diabetes, diarrhoea and pulmonary troubles. It is also used as diuretics, pain-killers, emetics, sedatives abortifacients, antiabortifacients, ecbolics, vermifuges, genital stimulants/degressants, etc. The aim of this study was to determine lethal dose concentration (LD_{50}), the effects of aqueous and ethanol leaf extracts of *E. psoraleoides* on the blood glucose level and some haematological parameters of alloxan-induced diabetic rats.

MATERIALS AND METHODS

Animals

A total of forty-eight albino mice of the male sex weighing 22 to 28 g were used for the acute toxicity study (LD_{50}). Twenty-eight adult Wistar rats weighing 180 to 220 g of the male sex were used for the anti-diabetes study. All animals were obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria. They were acclimatized for seven days in the Department of Biochemistry Animal house before the experiments and maintained *ad libitum* on water and growers mash (vital feeds).

Plant

The plant materials (leaves) were collected from Eha-Ndiagu in Nsukka Local Government Area of Enugu State, Nigeria. Botanical identification and authentication were performed by Mr. Ozioko of the International Center for Ethno-medicine and Drug Development Nsukka, Enugu State, Nigeria, where a herbarium sample with voucher specimen number Intercedd/16170 was prepared and deposited.

Chemical and drugs

All chemicals and reagent used in this study were of analytical grade. They included Alloxan monohydrate (Sigma-Aldrich, St. Louis, USA), Glibenclamide (Aventis Pharma Ltd., Verna, Goa), Tween 80 (S.D. Fine-Chem limited, Mumbai), Accu-chek® Active Glucometer, Roche Diagnostic Corporation, Germany, and blood gluco-strips (Roche Diagnostic Pvt. Ltd., Mumbai, India). Standard commercial test kits were also used for the study.

Extraction procedure

The aqueous extract of *E. psoraleoides* leaves was obtained by macerating 200 g of the powdered leaves in 800 ml water for 24 h with intermittent shaking. Following filtration through Whatman No. 1 filter paper, the filtrate was freeze-dried to obtain solid residue. The yield was recorded. The extract was reconstituted in distilled water in appropriate concentration before administration.

The ethanol extract of *E. psoraleoides* leaves was prepared by soaking 200 g of the powdered leaves in 800 ml of 95% ethanol for two days. The crude extract was evaporated to dryness *in vacuo* on a rotary evaporator. The yield was recorded and used in this study.

Phytochemical analyses

Preliminary phytochemical analyses of the plant extracts were performed for the presence of secondary metabolites, using the methods of Harborne (1984) and Evans (1996).

Acute toxicity (LD_{50}) studies

The LD_{50} determination for each of the extracts was conducted separately using modified method acute toxicity testing (Lorke, 1983). For each of the extracts, the evaluation was done in two phases. In phase one, three groups of three mice each were treated with 10, 100 and 1000 mg extract/kg body weight orally, respectively. The control groups received normal saline and 3% Tween 80. The rats were observed for clinical signs and symptoms of toxicity within 24 h and death within 72 h. Based on the results of phase one for the aqueous extract, 15 fresh mice with three per group were each treated with 1600, 2900 and 5000 mg extract/kg orally, respectively. The control groups received normal saline and 3% Tween 80. Clinical signs and symptoms of toxic effects and mortality were then observed within 24 h and death within 72 h. The LD_{50} were then calculated as the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase.

Induction of experimental diabetes mellitus

The animals were fasted for 16 to 18 h with free access to water prior to the induction of diabetes. Induction of diabetes was carried out by single intraperitoneal injection of alloxan monohydrate (Sigma St Louis, M.O., USA) dissolved in 0.9% v/v normal saline solution at a dose of 130 mg/kg body weight. The diabetes was assessed in alloxan-induced rats by determining the blood glucose concentration 72 h after injection of alloxan monohydrate. The rats with blood glucose level above 200 mg/dl were then selected for the study.

Experimental design

A total of twenty-eight rats were used and were divided into seven groups:

Group 1: Control (normal rats not treated with extract);
Group 2: Positive control (alloxan-induced diabetic rats not treated with extract);
Group 3: Alloxan-induced diabetic rats given 200 mg/kg body weight aqueous leaf extract of *E. psoraleoides*;
Group 4: Alloxan-induced diabetic rats given 400 mg/kg body weight aqueous leaf extract of *E. psoraleoides*;
Group 5: Alloxan-induced diabetic rats given 200 mg/kg body weight ethanol leaf extract of *E. psoraleoides*;
Group 6: Alloxan-induced diabetic rats given 400mg/kg body weight ethanol leaf extract of *E. psoraleoides*;
Group 7: Alloxan-induced diabetic rats given 0.3 mg/kg body weight glibenclamide (standard control).

On day 7, the rats were anaesthetized at the time of sacrifice by being placed in sealed cotton wool soaked chloroform inhalation jar.

Determination of blood glucose concentration

Fasting blood glucose levels were determined by using the glucose oxidase method with Accu-check glucometer (Activie) and results were reported as mg/dl.

Determination of haematological parameters

After treatment with the two extracts, 2 ml blood was withdrawn from the media canthus of the eyes of the rats by occipital puncture using heparinized capillary tube and the haemoglobin concentration (Hb), red blood cells (RBC), white blood cell count (WBC) and its differential counts were determined using the method of Ochei and Kolhaktali (2008), Cheesbrough (2000) and Ramnik (2003).

Statistical analysis

All the data are expressed as mean ± standard error of mean (SEM). Statistical comparisons were performed by one way analysis of variance (ANOVA) with repeated measures and one-way ANOVA followed by Duncan's multiple range tests (Duncan et al., 1977). The results were considered statistically significant if the values are 0.05 higher or lower.

RESULTS

Acute toxicity studies

In the investigation of acute toxicity, there was no sign of toxicity and mortality in the first phase of the study in both the aqueous and ethanol leaf extracts of *E. psoraleoides* but recorded the death in the second phase in the group that received 5000 mg/kg body weight of ethanol extract. The LD_{50} was then calculated as the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase. The LD_{50} of the AE and EE were thus:

$\sqrt{5000 \times 5000}$ = 5000 mg extract/kg body weight for AE

$\sqrt{2900 \times 500}$ = 3807.87 mg extract/kg body weight for EE

The acute toxicity (LD_{50}) value of ethanol leaf extract of *E. psoraleoides* leaves was calculated to be 3807.87 mg/kg body weight. The result of the oral acute toxicity (LD_{50}), studies showed aqueous and ethanol leaf extract of *E. psoraleoides* to be lethal at does 4000 and 5000 mg/kg body weight, an indication that the LD_{50} of the plant is less than 4000 mg/kg for EE and greater than 5000 mg/kg for AE. This result places *E. psoraleoides* at category 5 (>2000 to 5000 mg/kg) according to the Globally Harmonized System of Classification and Labeling of Chemicals (2013).

Effects of daily doses of aqueous and ethanol leaf extracts of *E. psoraleoides* on blood glucose concentration of alloxan-induced diabetic rats

Table 2 shows that the extracts and glibenclamide produced significant ($P<0.05$) decrease in the blood glucose concentration of alloxan-induced diabetic rats on day 7 when compared with group 2 (diabetic untreated). The mean differences are all greater than the LSD (52.60) for means separation of groups. The mean differences between group 1 and that of groups 3 and 4 do not exceed the LSD (52.60) on day 7. On day 6, only the extracts of *E. psoraleoides* produced significant ($P<0.05$) decrease in the blood glucose concentration of the alloxan-induced diabetic rats when compared with that of group 2 (diabetic untreated) which shows, they have close effect on the blood glucose concentration. The table also shows that in groups 3, 4, 5, 6 and 7, there is significant ($p<0.05$) decrease in the blood glucose concentration of the rats on days 6 and 7 when compared with that of days 4 and 5 as can be inferred from the LSD (21.41) for means separation of days.

Effects of daily doses of aqueous and ethanol leaf extracts of *E. psoraleoides* on haematological effect in alloxan-induced diabetic rats

Table 3 shows that the extracts of *E. psoraleoides* produced significant ($P<0.05$) change in red blood cell (RBC) count, white blood cell (WBC) count, neutrophils, lymphocytes and monocytes; however, they do not have significant ($P>0.05$) effect on haemoglobin. Only 400 mg/kg body weight ethanol extract of *E. psoraleoides* significantly ($P>0.05$) increased RBC in the rats when compared with the rats in group 2. The WBC of the rats treated with aqueous extracts decreased significantly ($P < 0.05$) when compared with that of group 2 (untreated group). All the treated rats showed significant ($P<0.05$) decrease in neutrophils and lymphocytes when compared with diabetic untreated rats. Also, rats treated with the four different doses of the extracts showed significant decrease in the neutrophils when compared with diabetic

Table 1. Phytochemical composition of aqueous and ethanol leaves extracts of *Eriosema psoraleoides*.

Sample	Concentration of phytochemicals in ethanol (mg/100 g)	Aqueous phytochemical concentrate (mg/100 g)
Soluble carbohydrate	2.13 ± 0.03	1.86 ± 0.03
Tannin	3. 45 ± 0.02	2.37 ± 0.02
Flavonoid	6.67 ± 0.20	5.86 ± 0.11
Cyanogenic glycoside	0.02 ± 0.01	0.01 ± 0.03
steroid	2.56 ± 0.15	2.47 ± 0.02
Alkaloids	8.66 ± 0.25	5.64 ± 0.25
saponin	0.57 ± 0.003	0.51 ± 0.03
Reducing sugar	262.47 ± 0.020	130.46 ± 0.06

rats treated with glibenclamide. Monocytes were not found in groups 2, 5 and 7. The groups where monocytes were found are groups 3, 4 and 6 and its levels were significantly low when compared with that of group 1 (Normal rats).

DISCUSSION

The effect of the aqueous and ethanol leaf extracts on rats' blood glucose has implications on its use for nutritional and therapeutic purposes. The observation that the extracts did not significantly cause a change in the glucose concentration of the normoglycemic rats after twenty four hours implies that the extract is safe in a normal subject taking it either as food or for other medical proposes. Apart from its safety in normoglycemic individuals, the extract has a high therapeutic index as the acute toxicity test in mice gave an LD_{50} of about 5000 mg extract/kg body weight for AE and 3807.87 mg extract/kg body weight for EE. It has been reported that flavonoids and tannins and allied phytochemicals present in plant's extracts possesses anti diabetic properties. Their compositions are shown in Table 1. Phytochemical analyses carried out revealed the presence of some bioactive compounds such as flavonoids, alkaloids, glycosides, steroids, reducing sugars, resins, tannins and saponins. The acute toxicity (LD_{50}) was 5000 and 2900 mg/kg body weight for aqueous and ethanol extracts of *E. psoraleoides*, respectively. The dosage of 130 mg/kg body weight of alloxan used in this study caused moderate diabetes (Grover et al., 2000).

As summarized in Table 2, the rats given alloxan showed hyperglycaemia effect across groups on day 3. The result agrees with already existing literature that alloxan induces diabetes mellitus by selectively destroying the beta cells of the pancreas which are involved in the synthesis of storage and release of insulin, the peptide hormone regulating carbohydrate, protein and lipid metabolism (Adeneye and Agbaje, 2008), leading to marked increase in blood glucose concentration observed in the rats after administration and confirms the development of diabetes mellitus (Akindele et al., 2012). The administration of aqueous and ethanol leaf extracts of *E. psoraleoides* for three days after induction of diabetes with alloxan significantly reduced ($p < 0.05$) this, probably meaning that the antidiabetic factors present in both extracts needed to be activated with time. It could also be that, the extracts may be facilitating the uptake of glucose by the peripheral cells. Alloxan induces type 1 diabetes (destruction of the β-cells that produce insulin) in which case the extracts may have some chemical components that exert regenerative effects on β-cells, stimulate these cells to start producing insulin (pancreatotrophic action) or may have some insulin-like substances and induction of regenerative stimulus in diabetic state triggers pancreatic regenerative processes, thereby restoring functional activities of the pancreas (Adewole and Ojewole, 2007). This observation gives credence to the use of the herbal product as a hypoglycemic agent.

The assessment of haematological parameters could be used to reveal the deleterious effect of foreign compound including plant extracts on the blood constituents of animal. They are also used to determine possible alterations in the levels of biomolecules such as enzymes, metabolic products haematology and normal function (Magalhaes et al., 2008). The occurrence of anaemia in diabetes mellitus has been reported to be due to the increased non-enzymatic glycosylation of RBC membrane proteins (Oyedemi et al., 2011). Oxidation of these proteins and hyperglycaemia in diabetes mellitus causes an increase in the production of lipid peroxides that leads to haemolysis of RBC (Arun and Ramesh, 2002). Diabetes mellitus causes the development of hypochronic anaemia due to a fall in the iron content of the body resulting from oxidative stress associated with the condition (Colak et al., 2012).

In this study, the RBC membrane lipid peroxide levels in diabetic rats were not measured. However, the red blood cells parameters such as Hb were studied to investigate the beneficial effect of *E. psoraleoides* leaf extracts on the anaemic status of the diabetic rats shown in Table 3. The extracts of *E. psoraleoides* produced

Table 2. Effect of AE and EE of *E. psoraleoides* on blood glucose concentration in alloxan-induced diabetic rats.

Day	Group 1 (Normal control)	Group 2 (Diabetic untreated)	Group 3 (Diabetes treated with AE (200 mg/kg))	Group 4 (Diabetes treated with AE (400 mg/kg))	Group 5 (Diabetes treated with EE (200 mg/kg))	Group 6 (Diabetes treated with EE (400 mg/kg))	Group 7 (Diabetes treated with glibendanide (0.3 mg/kg))
Day 1	86.25±8.26	86.25±6.02	83.75±9.81	89.00±6.38	94.75±16.40	100.75±10.63	90.75±14.41
Day 2	89.50±6.03	102.00±9.70	115.50±5.07	123.75±9.65	114.50±15.15	117.00±12.08	131.00±7.10
Day 3	92.50±12.58	265.00±8.44	360.75±5.63	320.25±6.26	378.75±8.83	252.75±8.07	313.25±9.29
Day 4	90.25±5.38	358.00±7.29	417.75±9.73	429.50±7.81	401.75±6.51	352.25±9.44	403.00±13.95
Day 5	93.50±6.45	369.00±5.52	347.00±9.02	334.00±10.00	315.50±9.54	326.50±9.91	351.75±11.70
Day 6	92.75±6.85	359.68±7.31	246.25±8.43*	271.75±15.76*	265.25±9.71*	268.50±9.43*	348.77±12.72*
Day 7	92.75±11.62	358.49±5.12	121.75±13.57*	128.75±13.98*	181.50±9.33*	192.25±5.74*	193.00±6.98*

AE: Aqueous extract; EE: ethanol extract; *E. Psoraleoides*: *Eriosema psoraleoides*. In all the groups n=4. Mean difference between any pair of days in a group greater than LSD value of 21.41 is significant at 5% level. Mean difference between any pair of groups on any day greater than LSD of 52.60 is significant at 5% level. p-value < 0.05 shows significant difference from Group 2.

Table 3. Effect of aqueous and ethanol leaf extracts of *E. psoraleoides* on some haematological parameters in alloxan-induced diabetic rats.

Parameter	Group 1 (Normal control)	Group 2 (Diabetic untreated)	Group 3 (Diabetes treated with AE (200 mg/kg))	Group 4 (Diabetes treated with AE (400 mg/kg))	Group 5 (Diabetes treated with EE (200 mg/kg))	Group 6 (Diabetes treated with EE (400 mg/kg))	Group 7 (Diabetes treated with glibendanide (0.3 mg/kg))
RBC × 10^9 L^{-1}	2.83±0.49^{a}	3.15±0.21^{ab}	3.23±0.24^{abc}	3.37±0.06^{bc}	3.65±0.07^{bc}	3.75±0.07^{c}	3.25±0.21^{abc}
Haemoglobin (g/dl)	12.87±1.27	14.18±1.17	12.60±0.93	13.23±0.35	13.40±0.70	14.35±0.49	13.10±0.14
WBC × 10^9 L^{-1}	6533.33±1001.67^{ab}	10425.00±3059.82^{c}	3737.50±717.79^{a}	8056.67±1336.65^{bc}	8460.00±1895.05^{bc}	8835.00±657.61^{bc}	7720.00±961.67^{bc}
Neutrophils (%)	24.67±5.03^{ab}	72.00±4.00^{d}	30.75±6.50^{b}	25.00±5.00^{ab}	15.00±0.00^{a}	32.00±16.97^{b}	45.00±0.00^{c}
Lymphocytes (%)	72.67±4.16^{cd}	27.50±3.79^{a}	68.75±7.46^{c}	74.33±4.04^{cd}	85.00±0.00^{d}	67.00±18.39^{bc}	55.00±0.00^{b}
Monocytes (%)	2.67±1.16^{b}	0.00±0.00^{a}	0.50±1.00^{a}	0.67±1.16^{a}	0.00±0.00^{a}	1.00±1.00^{ab}	0.00±0.00^{a}

AE: Aqueous extract; EE: ethanol extract; RBC: red blood cell count; WBC: white blood cell count. In the groups n = 4. Groups with different superscript(s) are significantly different from each other at 5% level.

significant (P < 0.05) change in red blood cell (RBC) count, white blood cell (WBC), neutrophlils, lymphocytes and monocytes. This observation is consistent with earlier reports (Mahmoud, 2013; Akomas et al., 2014; Chinyelu et al., 2017), but differ from the reports of some others (Mohammed et al., 2009; Verma et al., 2012).

However, the extract did not have significant (P>0.05) effect on haemoglobin. Only 400 mg/kg body weight ethanol extract significantly (P<0.05) increase RBC when compared with the diabetic untreated rats. The alterations of these parameters are well known to cause anaemic condition in man (Balasubraimanian et al., 2009). The white blood cell count of the rats treated with200 mg/kg body aqueous extract had significantly (P<0.05) decreased. These may be attributed to infection on the normal body systems of the rats. Also, a significant (P<0.05) decrease was observed in neutrophils and lymphocyte counts when compared with diabetic untreated.

The presence of some phytochemicals with the ability to stimulate the production of white blood cells in the extract could be responsible for the

observed result in the treated rats. The extract at both dosages significantly improved the levels of WBC and lymphocytes as well glibenclamide when compared with diabetic untreated group. The neutrophils increased significantly in the glibenclamide group as compared to the normal and extracts groups which had same effect. However, the extract did not have any significant effect on monocytes in this study. This gives an indication that the plant extracts may contain some phytochemicals that can stimulate the formation or secretion of erythropoietin in the stem cells of the animals. Erythropoietin is a glycoprotein hormone which stimulates stem cells in the bone marrow to produce red blood cells (Ohlsson and Aher, 2006). The RBC and Hb parameters are used mathematically to define the concentration of haemoglobin and to suggest the restoration of oxygen-carrying capacity of the blood.

Following the data obtained, it is suggested that aqueous and ethanol leaf extracts of *E. psoraleoides* possesses antihyperglycemic properties. In addition, the extract could prevent various complications of diabetes as well as improving some hematological parameters. A further experimental investigation is also needed to exploit its relevant therapeutic effect to substantiate its ethnomedicinal usage on macromolecules.

Conclusion

This investigation was anchored on the antidiabetic properties of *E. psoraleoides* to be compared with the standard drug glibenclamide. Though glibenclamide was more efficacious, going by the safety of herbal therapy, *E. psoraleoides* application still falls within the ambit of antidiabetic medicament. Its phytochemicals are testimonies of its activities and potency and it is strongly recommended for supplementation as food and treatment of type 2 diabetes mellitus.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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