Journal of Natural Products Discovery

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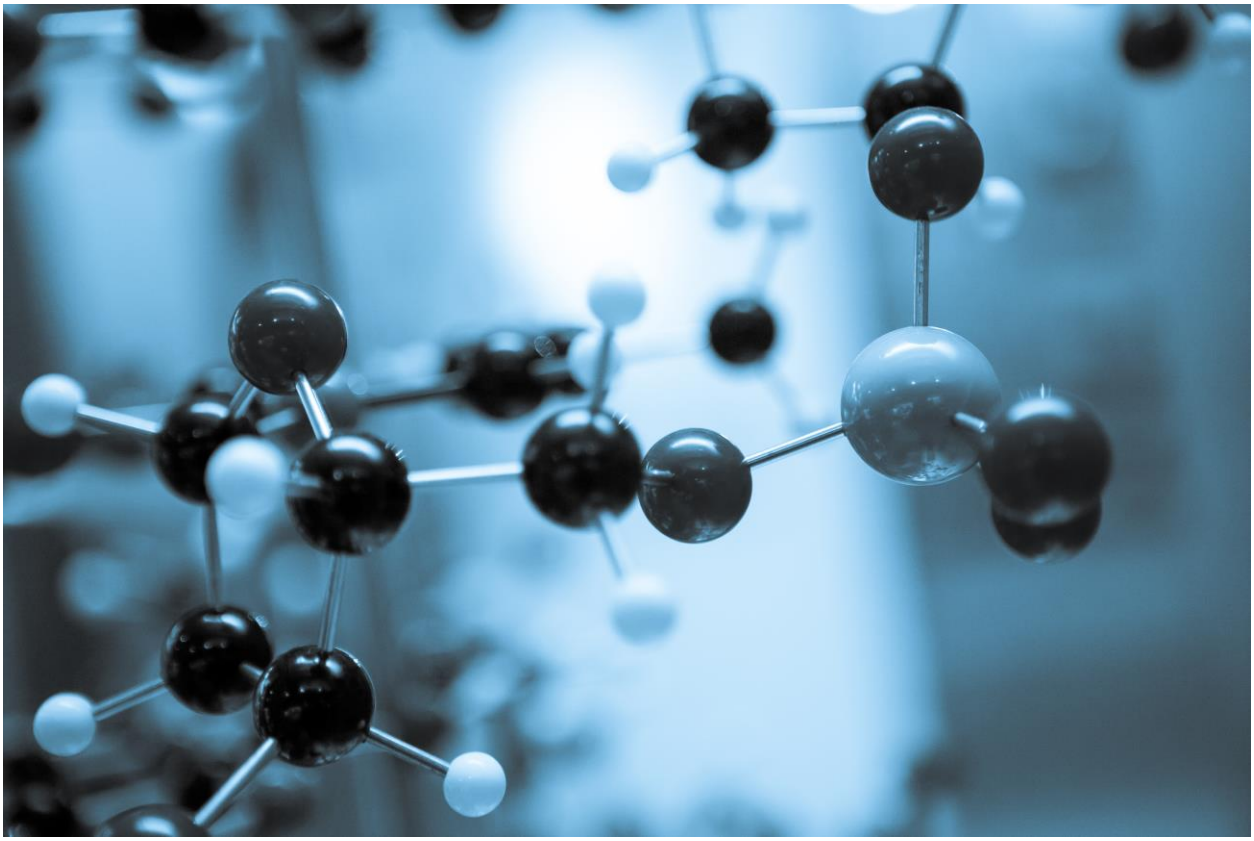

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JOURNAL OF NATURAL PRODUCTS DISCOVERY (JNPD)

is devoted to the publication of original articles The Journal publishes papers describing significant novelty in the analysis, isolation of metabolites, biological and pharmacological properties of natural products (including whole plants, marine organisms, terrestrial animals, fungi, bacteria and viruses, their cells, tissues and organs. The derived extracts and isolates should have characteristics or activities relevant for either basic biological sciences (botany, ecology, biochemistry, pharmacology, etc.) or use in the food, agrochemical, pharmaceutical, cosmetic and related industries. All forms of physical, chemical, biochemical, spectroscopic, radiometric, electrometric, chromatographic, metabolomic and chemometric investigations of plant products are also within the scope of the journal. Papers dealing with novel methods relating to areas such as data handling / data mining in plant sciences will also be welcomed.

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Journal of Natural Products Discovery

2022, Volume 1, Issue 1, pages 1, DOI 10.24377/jnpd.article660

Editorial

JOURNAL OF NATURAL PRODUCTS DISCOVERY (JNPD): COVERING NATURAL PRODUCTS RESEARCH FROM A NEW PERSPECTIVE

Natural products have long been utilized as a source of food, medicine and other commercially important materials and products, and because of this, natural products, from plants, microbes or animals, have been studied extensively in the ever-ending search for new products, particularly new drug candidates. Historically, several 'block buster' drugs like the anticancer drugs taxol, vincristine and vinblastine, antibiotics like penicillins and cephalosporins, and the narcotic analgesic morphine were discovered from natural sources, either from plants or microbes.

Over the last couple of decades, particularly since the appearance of the 'open access' platform, the number of journals that claim to cover research in the area of natural products has mushroomed. However, with the exception of a few reputed journals in this area, most of the other journals have become just a business venture, they charge large sum of money from the authors in the name of so called 'page charge', 'processing charge' or 'publication charge', and publish almost anything and everything without assessing the quality of publications. Most often, all these publications are repetitive, the same findings in a new package or even shamelessly plagiarised from previously published articles and have little or no applied scientific values. Moreover, most of the journals catering for natural products research cover mainly academic research without any tangible outcomes or discoveries that can lead to product development.

Journal of Natural Products Discovery (JNPD), which is the official journal of the Centre for Natural Products Discovery (CNPD) at Liverpool John Moores University (LJMU), will stand out from the crowd because of its product-discovery-focussed approach. The coverage will be focussed more to discoveries of new products or new applications of already known natural products in relation to food, pharmaceuticals, cosmetics and other commercial products.

The Centre for Natural Products Discovery (CNPD) was founded within the School of Pharmacy and Biomolecular Sciences at LJMU about three years ago with the aim to facilitate world-class research in natural products discovery impacting on societal, national and global needs. This centre has already organised successfully two international conferences in 2020 and 2021, and the third anniversary conference will be held in March 2022. A brand-new MSc programme in Natural Products Discovery was launched last year, as a part of the initiatives from CNPD to embed natural products research and to disseminate knowledge at various levels. Similarly, a monthly research newsletter was also launched a year ago to capture various activities of the centre. Publication of this new journal will further strengthen the activities of the centre and this journal will become one of the main outlets for communicating our research activities to a wider audience using the open access platform, sponsored by the LJMU Library.

In this connection, we gratefully acknowledge the outstanding support and assistance that we have received from Ms Catherine Dishman to establish this platform and set up the journal online submission system. We would also like to express our gratitude to our colleague Dr Jose Prieto Garcia (*Executive Editor-in-Chief*), who worked closely with Catherine, all other colleagues of the CNPD and us to establish this journal, and to make this very first issue possible. This journal is in open access, and there is no charge whatsoever levied to the authors, meaning the publication in this journal is completely free!

We hope this journal will grow rapidly as one of the leading journals in the field of natural products research and will attract the finest quality publications from researcher from all over the world.

Satyajit D. Sarker & Prof Khalid Rahman

Editors-in-Chief

Journal of Natural Products Discovery

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Letter to the Editors

NATURAL PRODUCTS DISCOVERY: CAVEATS, CONSIDERATIONS, COLLABORATION, CREATIVY AND CONTEMPORARY TECHNOLOGY

Dear editors,

There exists an enormous global need for new, safe, and effective drugs for a broad range of chronic and acute diseases. Most of these exigencies will not be met by “big Pharma”, ever. Consequently, in many countries, for a healthier and more productive population this pharmaceutical “chasm” must be bridged through alternative discovery efforts. At a time when environmental consciousness is increasing, and oil and coal processing for solvents and chemicals will likely be diminished in the future, synthetic pharmaceuticals cannot be relied on to serve as that bridge for the long-term. Natural products have continued to be a steady source of pharmaceuticals in the past 30 years (Newman & Cragg, 2020). However, a more emphatic, structured, and sustainable return to nature, using the contemporary technologies now available, is by far the best option to address global needs (Daley et al., 2021). This brief letter offers some general thoughts on natural product drug discovery programs and their initiation, operation, and development. It is not an exhaustive assessment, neither are the issues raised fully discussed, thus only a few key references are cited. The intention is to offer a background framework for scientists interested in this vast area of natural product research as they consider conducting their programs and publishing in this new and exciting journal. Five general areas of research development are presented: Caveats, Considerations, Collaborations, Creativity, and Contemporary Technology.

A. CAVEATS

i) The discovery and development of a drug, even to the stage of licensing, typically takes several years and considerable investment. Are the resources available to support that intended discovery pathway commitment? Do you have that level of personal persistence? Are there alternative pathways for consideration and approval which would bring drugs to the patient in a shorter, cheaper timeframe, while also maintaining safety and efficacy?

ii) Broad-based drug discovery from natural sources is always a series of compromises based on financial support for information systems, taxonomy, chemistry, and biology.

iii) Extensive background research is needed before a niche program can be developed to avoid wasteful duplicative studies.

iv) Is your research program based on a one-off study of an organism or part of a larger screening program? The intentions and therefore the strategies are completely different.

v) A significant number of compounds with a diverse taxonomic distribution are known to have a wide range of biological activities and may give false positive biological responses. These are IMPS and PAINS and must be identified early in the discovery process to avoid squandering isolation resources (Baell, 2016; Bisson et al., 2016).

vi) Artificial intelligence (AI)-assisted dereplication can identify these interfering compounds, along with other known bioactives, for a particular bioassay. An informatics-chemo-bioassay-linked system is an essential component for initial resource assessments in a drug discovery program.

vii) Marine and terrestrial organisms are typically intimately associated with pathogenic microorganisms, which themselves produce a range of potent metabolites. These may interfere in assay assessment by giving a non-reproducible false positive result, based on collection locale, or they may constitute a “hit” themselves.

B. CONSIDERATIONS

i) Awareness of both short and potentially long-term sustainable sourcing of research materials is essential. Avoid plant bark and slow generating roots for extraction. In laboratory practices minimize the application of heat and non-reusable solvent mixtures. Chromatographic supplies and glassware usage should be minimized. Consider additive manufacturing as a pathway to generate purpose-driven extraction and reagent vessels.

iii) Patenting considerations may push priorities towards new bioactive compounds. “Old” compounds with strong, selective, new bioactivities may, based on prior safety assessments, offer a significant advantage to new compounds in the IND approval process.

iv) Purified metabolites and partially processed extracts of sustainable resources are precious. Think small for sample size testing. Think large for test sample accumulation (collaborate locally and regionally to develop these assets), and for the breadth of bioassays applied to a given sample.

v) New research directions may require new methods. Training of personnel may be necessary for instrumentation and for bioassays.

vi) Geography matters. The same plant from the same location will likely have a modulated metabolic profile in a different season and/or from a different locale. If large compound samples are needed, a strategy for metabolite sustainability is necessary. Climate change in the location of origin will affect metabolic profile, requiring metabolomics and molecular networking studies as integral to the program.

vii) As a taxonomist know accurately what organism is being acquired and processed. As a chemist know precisely (profile and purity) what is being sent for biological assessment, and as a biologist know, at the time of the test, what is being evaluated.

viii) You will not have discovered the cure for cancer or diabetes. Do not suggest that based on one or two assays you have a potential therapeutic agent. Be hypercritical of relative activity and how the biological data are presented in a comparative manner.

C. COLLABORATIONS

i) Drug discovery programs, even limited ones, require collaboration between several disciplines. Quality, intensity, reliability, and consistency are necessary for success.

ii) Before starting, the research collaborations should be supported by negotiated agreements for sharing of intellectual property rights (IPR), compensation strategies for resources access, and authorship of publications.

iii) Based on the countries of sample acquisition, the IPR issues must be negotiated, and local approvals obtained. Time should be allowed for this essential phase in program development

iv) A compound of biological interest should be protected by the institution and then licensed depending on the intended application globally. Think open access for future development in different locations.

D. CREATIVITY

i) Robust, cheap, sensitive, rapid assays that target new mechanisms and approaches are continuously needed. Care is needed in selecting the appropriate positive control.

ii) Discovery includes studies potentiating synergistic relationships, and adjuvants which can deter destructive metabolic, or transport processes related to drug resistance.

iii) Research techniques for discovery are changing rapidly. Continuous program assessment is essential. If you are doing the same science as 5 years ago, something is fundamentally wrong. Know when to stop a program, or aspects of it, to protect resources.

iv) Opportunity may arise from unexpected results; stay aware for the serendipitous outcome.

v) Translation of research has been transformed in the recent past, and even more so with COVID-19 research. The traditional avenues of peer review and publication are changing. Where, how, and when results are published or protected is a collaborative discussion.

E. CONTEMPORARY TECHNOLOGY

i) Every researcher has intimate access to comprehensive information systems and to highly specialized databases. How these assets are used creatively determines the relevance, the focus, and the success of the program. Innovative discovery programs will evolve through integrating the technologies embodied in the Fourth Industrial Revolution and fostering collaborations with the government and industry to address program needs. Considering the relevant sustainable development goals and creating a balance between machine learning, in silico metabolite suggestions, robotic processing of extracts, network pharmacology, and the human interpretation of AI-generated outcomes, are critical elements of an integrated discovery program.

ii) Secure and immutable research data recording is essential, particularly if intellectual property rights and potential patent claims are to be protected.

iii) Where is the primary discovery of an organism of interest being made? Is it in the laboratory or in the field ("ecopharmacognosy in a suitcase")? Microfluidic biosensors for discovery of extracts to be pursued for in-field and in-laboratory use are an important asset for program enhancement.

iv) The ability to define diverse, "silent" biosynthetic gene clusters in fungal and bacterial genomes stimulates their activation as discovery havens for illuminating new ranges of metabolites.

v) Applications of synthetic biology abound for natural product discovery programs, notably for enhancing structure diversity through new operon construction, optimizing biosynthetic pathways, discerning drug targets, and disclosing new enzymes for processes that are chemically and energetically non-sustainable, or use expensive, non-recyclable reagents.

The many ways forward for natural products in drug discovery are clear and the opportunities for creative and meaningful discoveries abundant. Crucial for progress for the patient is high level, focused, dedicated collaborative programs which realize that success involves government, industry, and academia coming together. Territories and egos must be set aside in support of research towards common human health goals of Quality, Safety, Efficacy, Consistency, and Accessibility (QSECA) for nature-derived medicinal agents to meet local and global needs (Cordell, 2019).

REFERENCES

Baell, J.B. (2016) Feeling nature's PAINS: natural products, natural product drugs, and pan assay interference compounds (PAINS). Journal of Natural Products 79(3), 616-628.
Bisson, J., McAlpine, J.B., Friesen, J.B., Chen, S.-N., Graham, J., and Pauli, G.F. (2016) Can invalid bioactives undermine natural product-based drug discovery? J. Med. Chem. 59(5), 1671-1690.
Cordell, G.A. 2019. Cyberecoethnopharmacolomics. Journal of. Ethnopharmacology 244, 112134.
Daley, S. K., & Cordell, G. A. (2021). Alkaloids in Contemporary Drug Discovery to Meet Global Disease Needs. Molecules (Basel, Switzerland), 26(13), 3800.
Newman, D.J. and Cragg, G.M. (2020) Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. Journal of Natural Products 83(3), 770-803.

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Review Article

THERAPEUTIC POTENTIAL OF *LEEA INDICA* (VITACEAE)

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ABSTRACT

Background

Leea indica (Burm. f.) Merr. (fam. Vitaceae), commonly known as 'bandicoot berry', is a Thai medicinal plant, and distributed widely in the far-east and south-east Asian countries, and in some parts of northern Australia. In Thailand, this plant has traditionally been used for the treatment of diarrhoea, pain, gastric ulcer, viral infections and some forms of cancers.

Aims

To review published findings on medicinal properties of *L. indica* and to critically appraise its therapeutic potential.

Methods

A comprehensive literature search was performed utilizing several databases, notably, Web of Science, PubMed and Google Scholar, and other relevant published materials. The keywords used in the search, individually as well as in combinations, were *Leea indica*, Vitaceae and traditional medicine.

Results

In vitro assays and *in vivo* animal studies displayed efficacy of the extracts and fractions of *L. indica* as an analgesic, antidiabetic, anti-inflammatory, antimicrobial, antioxidant and antiproliferative agent and indicated their therapeutic potential. Phytochemical studies revealed the presence of alkaloids, flavonoids, polyphenolics and terpenoids as major bioactive components in *L. indica*.

Conclusion

Preliminary bioactivity studies on *L. indica* provided some scientific basis for its traditional therapeutic applications. The presence of certain bioactive compounds in this plant could further support its therapeutic potential and traditional medicinal uses.

Keywords: *Leea indica*, Vitaceae, anticancer, antioxidant, traditional medicine.

INTRODUCTION

Leea indica (Burm. f.) Merr., commonly known as 'bandicoot berry', is a Thai medicinal plant from the family Vitaceae, and distributed widely in the far-east and south-east Asian countries, *e.g.,* Bangladesh, China, India, Malaysia, Nepal, Sri Lanka and Vietnam, and in some parts of northern Australia (Wong and Kadir, 2011; Singh et al., 2019a,b). This species is an evergreen perennial shrub or a small tree growing up to 2-16 m tall (Figure 1).

a.  b.  c. 

Figure 1. Stipule (a), flowers (b) and unripe fruits (c) of *Leea indica*

In Thailand, this plant has traditionally been used for the treatment of diarrhoea, gastric ulcers, leucorrhoea, pain, viral infections and some forms of cancers (Mollik et al., 2009; Wong et al., 2012; Mishra et al, 2016; Kekuda et al., 2018; Singh et al., 2019b). The roots are used for treating diarrhoea, dysmenorrhoea fever and muscular pain; roots and stem are consumed for the treatment of diarrhoea, gastric ulcers and haemorrhoid; young shoots are applied externally to reduce swelling; stipules are used to treat *Herpes* infections (Kekuda et al., 2018; Singh et al., 2019b). Furthermore, this plant is used as an ingredient in traditional medicinal preparations to treat leucorrhoea, intestinal and uterus cancers (Wong et al., 2012), and the roots are used as an aphrodisiac and neurotonic remedy in Thai traditional medicines (Temkitthawon et al., 2011). Because of its long-standing contributions to Thai traditional medicinal practice as well as in traditional medicines from various other countries, this plant has been the subject of phytochemical and pharmacological studies providing some scientific evidence for its applications. This review article explores published findings on medicinal properties of *L. indica*, and critically appraises its therapeutic potential.

PHYTOCHEMISTRY

Previous phytochemical analyses of *L. indica* furnished the presence of various plant secondary metabolites belonging to the phytochemical classes of alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins and terpenoids (Table 1). However, many of these studies were merely qualitative phytochemical screening without isolation and identification of phytochemicals.

Alkaloids

Alkaloids are nitrogenous compounds where the nitrogen is usually an integral part of the ring, and they form one of the largest groups of plant secondary compounds with a variety of pharmacological activities (Nahar and Sarker, 2029). In fact, several modern natural products derived drugs belong to this class of natural products, *e.g.,* anticancer drugs vincristine and vinblastine from *Catharanthus roseus*, narcotic analgesic morphine from *Papaver somniferum*, and so on. Previous phytochemical studies revealed the presence of alkaloids in the leaves and stem bark of on *L. indica* (Emran et al., 2012a,b; Rahman et al., 2013a,b; Dalu et al., 2014; Mishra et al., 2014; Harun et al., 2016, 2018; Chander and Vijayachari, 2016; Tareq et al., 2017; Ghagane et al., 2017). Emran et al. (2012a,b) established the presence of alkaloids in the leaves of this plant based on qualitative tests for alkaloids, but no isolation of specific alkaloids was attempted. Several other authors also reported the presence of alkaloid in this plant merely on the basis of preliminary qualitative tests (Rahman et al., 2013a,b; Dalu et al., 2014; Mishra et al., 2014; Chander and Vijayachari, 2016; Harun et al., 2016; Tareq et al., 2017). The qualitative test conducted by Harun et al. (2016) identified alkaloids in the leaves extract, but not in the extracts obtained from the stem and roots. However, later this group tentatively identified the alkaloid 3,8,8-trimethoxy-3-piperidyl-2,2-binaphthalene1,1,4,4-tetrone (Figure 2) in the leaves and stem, based on GC-MS analysis (Harun et al., 2018), but this alkaloid was not isolated. Notably, Ghagane et al. (2017) did not find the presence of alkaloids in *L. indica* from the qualitative tests for alkaloids that they performed. Discrepancies in the outcomes of qualitative tests conducted by various groups could certainly raise the questions about their reliability and precision, and points to the need for proper separation, isolation and identification of individual phytochemicals present in various extracts.

Table 1| A summary of phytochemistry of *L. indica*

Phytochemical classes	Plant parts	References
Alkaloids	Leaves and stem bark	Emran et al., 2012a,b; Rahman et al., 2013a,b; Dalu et al., 2014; Mishra et al., 2014; Harun et al., 2016, 2018; Chander and Vijayachari, 2016; Tareq et al., 2017; Ghagane et al., 2017
Alkanes	Leaves	Srinivasan et al., 2008
Alkenes	Leaves and stem	Srinivasan et al., 2008; Harun et al., 2018
Cardiac glycosides	Leaves, roots and stem	Rahman et al., 2013a,b; Dalu et al., 2014
Carotenoids	Leaves	Singh et al., 2019a,b
Coumarin	Leaves	Singh et al., 2019a,b
Dihydrochalcones	Leaves	Singh et al., 2019a
Fatty acids	Leaves and stem	Harun et al., 2018; Singh et al., 2019a,b; Baharom et al., 2020
Fatty alcohols	Leaves	Srinivasan et al., 2008; Baharom et al., 2020
Flavonoids	Leaves	Emran et al., 2012a,b; Joshi et al., 2013; Rahman et al., 2013a,b; Mishra et al., 2014; Chander and Vijaychari, 2016; Harun et al., 2016; Ghagane et al., 2017; Patel et al., 2017; Tareq et al., 2017; Singh et al., 2019a,b
Glycosides	Leaves and stem	Rahman et al., 2013a,b; Ghagane et al., 2017; Tareq et al., 2017
Megastigmane	Leaves	Singh et al., 2019a
Phthalic acid esters	Flowers, leaves and roots	Joshi et al., 2013, Srinivasan et al., 2008, 2009
Polyphenolic compounds	Leaves, roots, stem bark and whole plant	Srinivasan et al., 2008; Emran et al., 2012a,b; Joshi et al., 2013; Rahman et al., 2013a,b; Dalu et al., 2014; Mishra et al., 2014; Harun et al., 2016; Ghagane et al., 2017; Patel et al., 2017; Tareq et al., 2017; Singh et al., 2019a
Saponins	Leaves, roots and stem	Dalue at al., 2014; Harun et al., 2016; Ghagane et al., 2017
Steroids	Leaves, roots and stem	Srinivasan et al., 2008; Emran et al., 2012a,b; Joshi et al., 2013; Rahman et al., 2013a,b; Dalu et al., 2014; Harun et al., 2016; Tareq et al., 2017; Singh et al., 2019a
Terpenoids	Leaves	Srinivasan et al., 2008; Emran et al., 2012a,b; Wong et al., 2012; Joshi et al., 2013; Dalu et al., 2014; Chander and Vijaychari, 2016; Harun et al., 2016, 2018; Singh et al., 2019a
Various	Leaves and stem	Harun et al., 2018

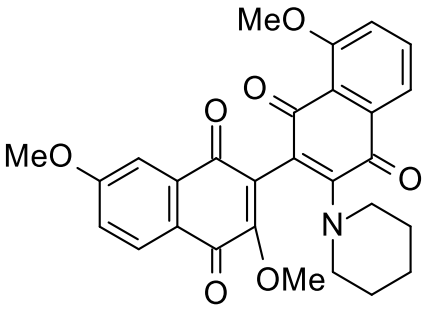


Figure 2. 3,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene1,1,4,4-tetrone in *L. indica*, identified by GC-MS

Alkanes

Alkanes occur ubiquitously in various plants and are often ignored or discarded by phytochemists when it comes to isolation of bioactive compounds from plant extracts. Srinivasan et al. (2008) identified a series of alkanes in a nonpolar extract of the leaves of *L. indica*, including *n*-eicosane, *n*-heptacosane, *n*-heptadecane, *n*-octadecane, *n*-tetracosane, *n*-tetratetracontane, *n*-tetratriacontane, *n*-tricosane and *n*-tritetracontance.

Alkenes

Like alkanes, long-chain alkenes are not normally the target compounds in any standard phytochemical isolation protocols. However, they are often identified by GC-MS analysis, usually from nonpolar extracts of plant materials. Srinivasan et al. (2008), and later, Harun et al. (2018) identified a couple of alkenes, *e.g.,* 17-pentatriacontene and 9-octadecene, from the extracts of the leaves and stem of *L. indica* by GC-MS analysis.

Cardiac glycosides

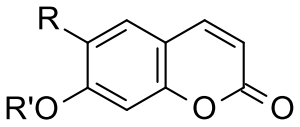
Dalu et al. (2014) and Rahman et al. (2013a,b) showed the presence of cardiac glycosides by preliminary qualitative tests for this type of compounds, but no cardiac glycosides have been isolated and identified from *L. indica* to date.

Carotenoids

Carotenoids are one of the widely distributed compounds in plants, and often possess a high degree of antioxidant properties (Nahar and Sarker, 2019). Sing et al. (2019a,b) detected carotenoids in different solvent fractions obtained from a methanolic extract of the leaves of *L. indica*, but no purifications for carotenoids have ever been reported for this plant.

Coumarins

Only one coumarin, esculetin (Figure 3), was reported from the leaves of *L. indica* based on LC-MS analysis (Singh et al., 2019a,b). The distribution of esculetin, like scopoletin, scoparone and umbelliferone, is quite widespread in the plant kingdom.



Esculetin R = OH, R’ = H; Scopoletin R = OMe, R’ = H;

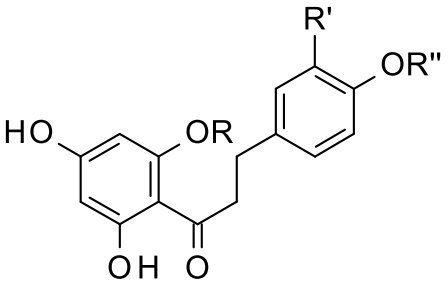
Scoparone R = OMe, R’ = Me; Umbelliferone R = R’ = H

Figure 3. Esculetin from the leaves of *L. indica*

Dihydrochalcones

Singh et al. (2019a) reported dihydrochalcone, 4’,6’-dihydroxy-4-methoxydihydrochalcone 2’-*O*-β-D-glucopyranoside (Figure 4), from the leaves of *L. indica*. A repeated column chromatography approach was adopted for the isolation of this dihydrochalcone, which was identified comprehensively by spectroscopic means. A few other dihydrochalcones, 3-hydroxyphloridzin, phloridzin, 4’,6’-dihydroxy-4-methoxydihydrochalcone 2’-*O*-rutinoside, 4’,6’-dihydroxy-4-methoxydihydrochalcone 2’-*O*-glucosylpentoside, 4’,6’-dihydroxy-4-methoxydihydrochalcone 2’-*O*-(3’’-

O-galloyl)-β-D-glucopyranoside and 2',4',6'-trihydroxy-4-methoxydihydrochalcone (3-methylphloretin) (Figure 4), were identified by LC-MS analysis without isolation.



4',6'-Dihydroxy-4-methoxydihydrochalcone 2'-*O*-β-D-glucopyranoside, R = Glucopyranosyl; R' = H; R'' = Me

3-Hydroxyphloridzin R = Glucopyranosyl; R' = OH; R'' = H

Phloridzin R = Glucopyranosyl; R' = R'' = H

4',6'-Dihydroxy-4-methoxydihydrochalcone 2'-*O*-rutinoside, R = Rutinosyl; R' = H; R'' = Me

4',6'-Dihydroxy-4-methoxydihydrochalcone 2'-*O*-glucosylpentoside, R = Glucosyl-pentosyl; R' = H; R'' = Me

4',6'-Dihydroxy-4-methoxydihydrochalcone 2'-*O*-(3''-*O*-galloyl)-β-D-glucopyranoside, R = 3-galloyl-glucosyl; R' = H; R'' = Me

2',4',6'-Trihydroxy-4-methoxydihydrochalcone (3-methylphloretin), R = R' = H; R'' = Me

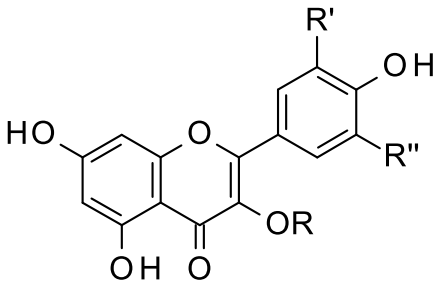
Figure 4. Dihydrochalcones from *L. indica*

Fatty acids and fatty alcohols

Like long-chain alkanes and alkenes, fatty acids and alcohols are quite widespread in the plant kingdom, and they are usually not the subject of isolation and identification in any standard phytochemical work, unless the work specifically aims to purify these compounds for any particular reason. Nonetheless, in the process of isolating other secondary metabolites, some of these fatty acids and alcohols are inadvertently isolated and identified. Also, GC-MS based analysis of plant extracts often reveals the presence of these compounds in the extract. Methyl stearate, oleic acid, 9,12-octadecadienoic acid, 9-oxononanoic acid, palmitic acid and 9,12,13-trihydroxy-octadecadienoic acid, were reported as the major fatty acids in the leaves and stem of *L. indica* (Srinivasan et al., 2008; Harun et al., 2018; Singh et al., 2019a), while 1-eicosanol and farnesol were detected as two main fatty alcohols in the leaves of this plant (Srinivasan et al., 2008).

Flavonoids

Flavonoids are a large group of phenolic compounds found in several plant families and possess various important medicinal properties (Nahar and Sarker, 2019). Emran et al. (2012a,b) established the presence of flavonoids in the leaves of this plant based on qualitative tests for flavonoids, but no isolation and identification of specific flavonoids was achieved. Similar qualitative result was obtained by a few other researchers (Rahman et al., 2013a,b; Dalu et al., 2014; Mishra et al., 2014; Chander and Vijayachari, 2016; Harun et al., 2016; Ghagane et al., 2017; Tareq et al., 2017). The presence of one of the most common flavonols, quercetin (Figure 5), in an ethanolic extract of the aerial parts of *L. indica* was confirmed by HPTLC analysis (Patel et al., 2017); previously this flavonol was isolated and identified from an ethanolic extract of the roots of this plant (Joshi et al., 2013). Sing et al. (2019a) isolated and identified two flavonoid glycosides, myricetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside from a methanolic extract of the leaves of *L. indica*, while identified several other flavonoids, including epicatechin, epigallocatechin, (-)-epigallocatechin 3-*O*-gallate, gallocatechin, kaempferol, kaempferol 3-*O*-arabinoside, kaempferol 3-*O*-rhamnoside, myricetin (2''-O-gllayl)-3-rhamnoside and quercitrin 2''-gallate, in this extract as a result of detailed LC-MS analysis (Figure 5).



Kaempferol R = R’ = R’’ = H

Kaempferol 3-*O*-arabinoside R = Arabinosyl, R’ = R’’ = H

Kaempferol 3-*O*-rhamnoside R = Rhamnosyl, R’ = R’’ = H

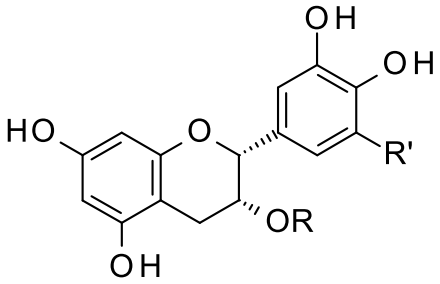
Myricetin 3-*O*-rhamnoside R = Rhamnosyl. R’ = R’’ = OH

Myricetin (2’’-*O*-gllayl)-3-*O*-rhamnoside R = (2’’-*O*-Galloyl)-3-*O*-rhamnosyl, R’ = R’’ = OH

Quercetin R = R’’ = H, R’ = OH

Quercetin 3-*O*-rhamnoside R = Rhamnosyl, R’ = OH, R’’ = H

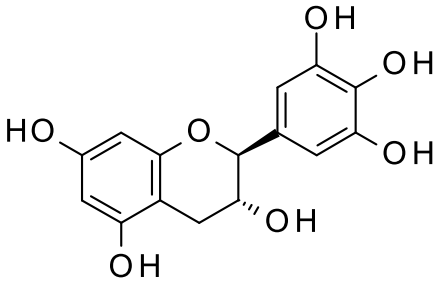
Quercitrin 2’’-gallate R = (2’’-*O*-Galloyl)-3-*O*-rhamnosyl, R’ = OH, R’’ = H



Epicatechin R = R’ = OH

(-)-Epigallocatechin R = H, R’ = OH

(-)-Epigallocatechin 3-*O*-gallate R = Galloyl, R’ = OH



Gallocatechin

Figure 5. Major flavonoids in *L. indica*

Glycosides

Several authors reported the presence of unidentified glycosides in the leaves and stem of *L. indica* (Rahman et al., 2013a,b; Mishra et al., 2014; Ghagane et al., 2017; Tareq et al., 2017) based on qualitative chemical assays for glycosides. Some of these glycosides were eventually identified as mainly dihydrochalcone and flavonoid based glycosides (Singh et al., 2019a) as shown in Figures 4 and 5.

Megastigmane

Megastigmanes represent a large group of C13 derivatives present in fruits and vegetables, as well as in non-edible plants (Nahar and Sarker, 2019). The only megastigmane that was identified to date in the leaves of *L. indica* is dehydrovomifoliol (Figure 6) (Singh et al., 2019a). However, no attempt was made to isolate and identify this compound from the extract.

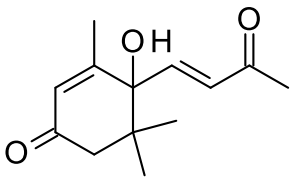


Figure 6. Dehydrovomifoliol, the only megastigmane reported from *L. indica*

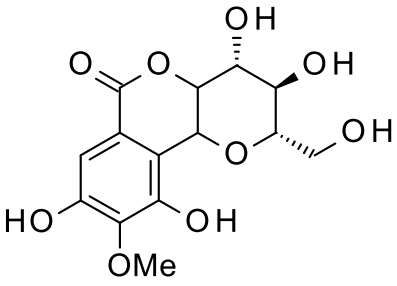
Phthalic acid esters

Phthalic acid derivatives, including esters, are not necessarily the desired components in phytochemical investigations. Nonetheless, phytochemists often end up with isolating or identifying these compounds in various plant materials. Most often, these compounds are the artefacts that come from various plastic containers, impure

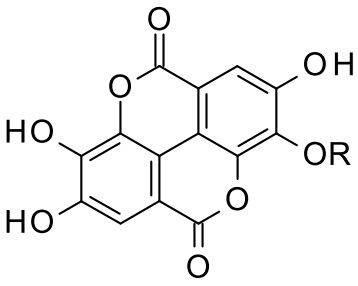
solvents and other sources that the plant materials come in contact with. Di-*n*-butyl-phthalate and di-*n*-octyl-phthalate were reported from the roots of *L. indica*, while di-isobutyl-phthalate, di-*n*-butyl-phthalate, *n*-butyl-isobutyl-phthalate and butyl-isohexyl-phthalate were found in the flowers (Srinivasan et al., 2009). Phthalic acid, di-*n*-butyl-phthalate, butyl-2-ethylhexyl-phthalate and isooctyl- phthalate were identified in the leaves of this plant (Srinivasan et al., 2008).

Polyphenols

Polyphenols constitute one of the largest groups of bioactive phytochemicals, with high antioxidant capacities (Nahar and Sarker, 2019). In addition to dihydrochalcones, flavonoids and their glycosides, which have already been discussed in the earlier sections, there are several other more complex polyphenolic compounds, often in the form of tannins. There are also several simple polyphenolic compounds found in various plants. Emran et al. (2012a,b) established the presence of polyphenolic compounds like tannins in the leaves of this plant based on qualitative tests for tannins, but no isolation and identification of specific tannins was pursued. Gallic acid and methyl gallate (Figures 7) are two most prevalent simple polyphenolic compounds found in various plant parts of *L. indica* (Joshi et al., 2013; Srinivasan et al., 2008; Patel et al., 2017; Singh et al., 2019a). α-Tocopherol (Figure 7), another well-known natural antioxidant, was found in the roots of this plant (Joshi et al., 2013), while bergenin, ellagic acid and methyl-*O*-ellagic acid (Figure 7) were identified in the leaves by LC-MS (Singh et al., 2019a). Theasinensin A isomers, theasinensin A quinone and theasinensin F (Figure 8) are among the more complex polyphenols reported from the leaves of *L. indica* (Singh et al., 2019a).

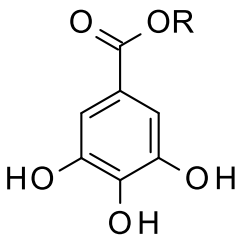


Bergenin



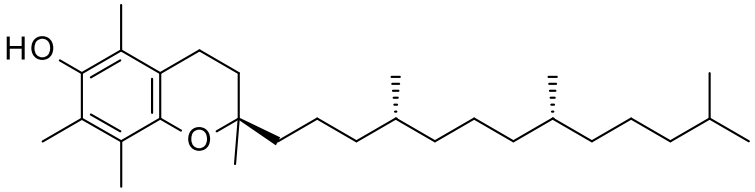
Ellagic acid R = H

Methyl-*O*-ellagic acid R = Me



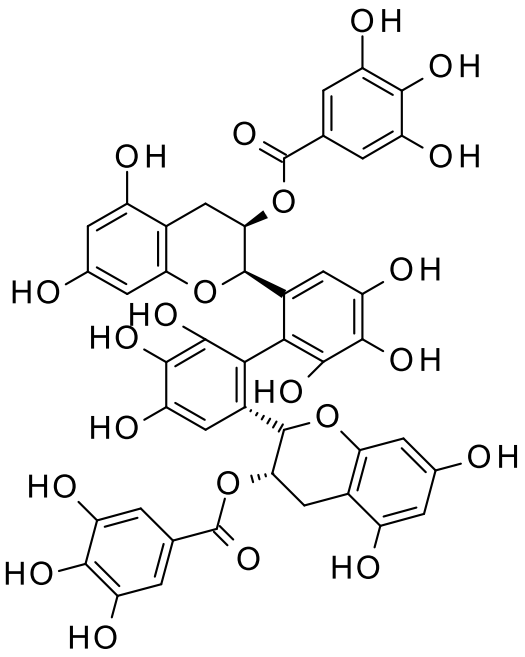
Gallic acid R = H

Methyl gallate R = Me

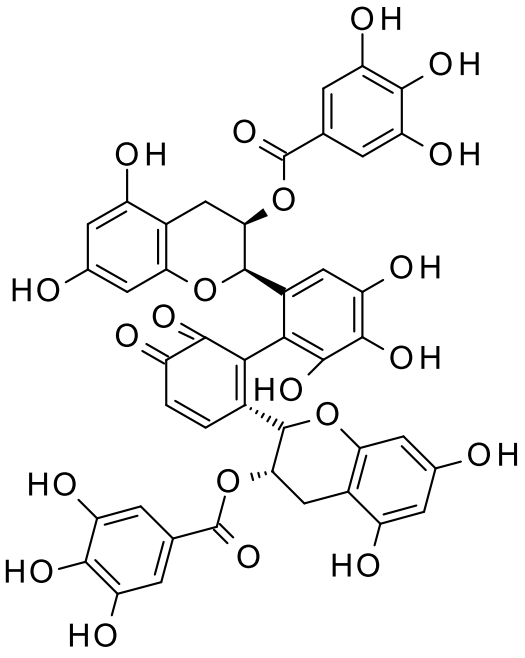


α-Tocophenrol

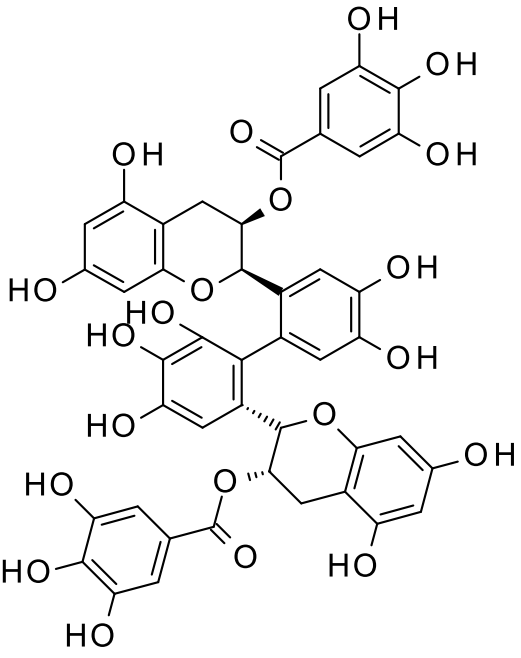
Figure 7. Simple polyphenolic compounds present in *L. indica*



Theasinensin A isomers



Theasinensin A quinone



Theasinensin F

Figure 8. Complex polyphenolic compounds present in *L. indica*

Saponins

A number of researchers (Rahman et al., 2013a,b; Dalu et al., 2014; Harun et al., 2016) showed the presence of saponins in the leaves, stem and roots of this plant based on qualitative tests for saponins, but no isolation and identification of specific saponins was performed. It is interesting though, that Emran et al. (2012b) did not detect the presence of saponins in the leaves when they performed the same qualitative test.

Steroids

Several researchers established the presence of plant sterols in the leaves of this plant based on qualitative tests for steroids, but no isolation and identification of specific steroids was achieved (Emran et al. 2012a,b; Rahman et al., 2013a,b; Dalu et al., 2014; Harun et al., 2016). Joshi et al. (2013) and Srinivasan et al. (2008) isolated β-sitosterol, which is the most distributed sterol in the plant kingdom, respectively, from the roots and leaves of *L. indica*. β-Sitosterol 3-*O*-glucoside was also reported from the roots of this plant (Singh et al., 2019a).

Terpenoids

Emran et al. (2012a,b) established the presence of terpenoids in the leaves of this plant based on qualitative tests for terpenes without isolation and identification of specific terpenoids. Several other authors also reported the presence of terpenoids based on qualitative tests (Joshi et al., 2013; Dalu et al., 2014; Chander and Vijaychari, 2016; Harun et al., 2016, 2018). However, a few researchers isolated and identified β-amyrin, *O*-hexadecanoyl-β-amyrin, lupeol, mollic acid α-L-arabinoside, mollic acid β-D-xyloside, 2α,3α,23-trihydroxy-12-oleanen-28-oic acid and ursolic acid from various plant parts of *L. indica* (Table 1) (Figure 8) (Srinivasan et al., 2008; Wong et al., 2012; Joshi et al., 2013; Singh et al., 2019a,b).

Various other compounds

Harun et al. (2018) reported the presence of 3,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone in the leaves, and 1-(hydroxymethyl)-1,2-ethanediyl ester in the stem of *L. indica*.

THERAPEUTIC POTENTIAL

Therapeutic potential of crude extracts and fractions

Pharmacological properties of the extracts and fractions of *L. indica* as established by several studies and published in the literature, are summarized in Table 2.

Analgesic activity

An ethanolic extract of the leaves of *L. indica* was found to exhibit central and peripheral analgesic effects in mice which could support its traditional uses in the management of pains. The assessment of the analgesic effect of this ethanolic extract was carried out by Emran et al. (2012a), who observed that the extract (200 mg/kg, p.o.) significantly ($p < 0.05$) inhibited the writhing response in acetic acid-induced writhing test compared to the known analgesic diclofenac sodium (40 mg/kg, i.p.). They also found that the extract could suppress the pain response (8.18%) in formalin-induced licking test compared to diclofenac sodium (66.45%; 0.5 mg/kg, i.p.).

Antiangiogenic activity

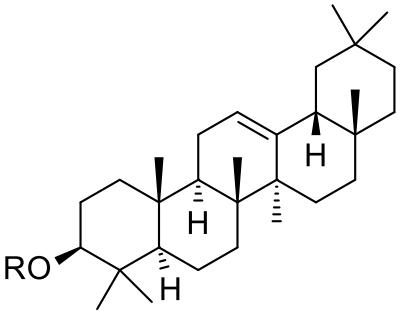
Recombinant vascular endothelial growth factor (rVEGF165) induced *in vivo* chorio alontoic membrane, rat corneal micropocket, and tumour-induced peritoneal angiogenesis assays were used for the preliminary screening of an ethanolic extract of the leaves of *L. indica*. It was found that the crude extract (50 mg/kg) could inhibit the sprouting vessels both in non-tumorigenic and tumorigenic conditions. Inhibition of VEGF expression by the extract contributed for tumour inhibitory effect. It was suggested that the presence of triterpenoids (Figure 8) in the extract might be a contributory factor for this angiomodulatory effect (Avin et al., 2014).

Antidiabetic activity

Antihyperglycemic activity of alcoholic and hydroalcoholic extracts of *L. indica* leaves was evaluated by the glucose tolerance test and alloxan-induced diabetes model in rats. Both extracts (200 and 400 mg/kg body weight) significantly decreased blood glucose level without displaying any acute toxicity (Dalu et al., 2014). As a result of oral treatment with the alcoholic and hydroalcoholic extracts (200 mg/kg and 400 mg/kg) for 21 days in alloxan-induced diabetic rats, the extracts were shown to have hypolipidemic activity by reducing triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, very low-density lipoprotein (VLDL) cholesterol and elevated high density lipoprotein (HDL) levels (Dalu et al., 2014). Another study demonstrated that a hydroalcoholic extract

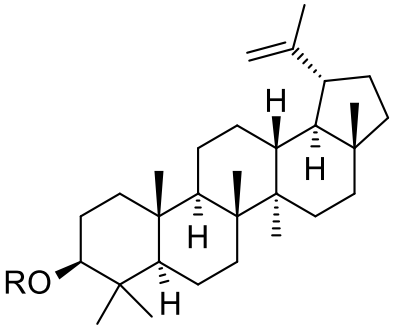
of *L. indica* leaves could significantly increase glucose uptake in isolated rat hemidiaphragm, improve glycogen content and inhibit α-glucosidase enzyme (Dalu and Dhulipala, 2016). A methanolic extract of *L. indica* leaves (200 mg/kg, p.o.) was found to reduce blood glucose levels in alloxan-induced diabetic rats (Patel et al., 2016). A methanolic extract of the leaves was accessed its effect on porcine pancreatic lipase activity, and it was observed that the extract could inhibit the activity of lipase by 48.5% (Ado et al., 2013).

It was suggested that the antihyperglycemic and hypolipidemic properties of *L. indica* could be due to the presence of ursolic acid (Figure 8) and gallic acid (Figure 7), as ursolic acid was reported as an effective insulin-mimetic agent and gallic acid was reported to be an insulin-secretagogue, antihyperlipidemic and antioxidant (Dalu et al., 2014 and Dalu and Dhulipala, 2016).

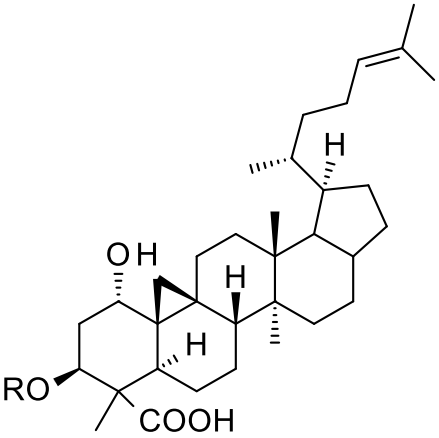


β-Amyrin R = H

O-Hexadecanoyl-β-amyrin R = Hexadecanoyl

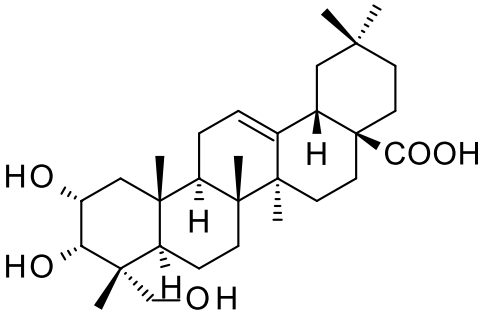


Lupeol

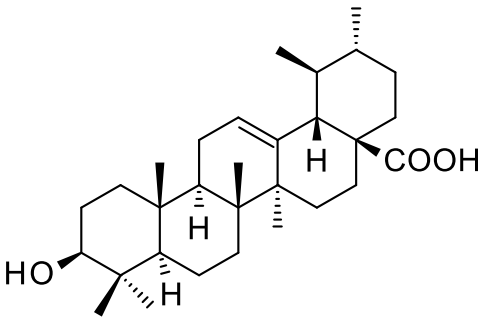


Mollic acid α-L-arabinoside R = Arabinosyl

Mollic acid β-D-xyloside R = Xylosyl



2α,3α,23-Trihydroxy-12-oleanen-28-oic acid



Ursolic acid

Figure 8. Major terpenoids present in *L. indica*

Table 2| Pharmacological properties of *L. indica*

Pharmacological properties	Plant parts	Extracting solvents	References
Analgesic	Leaves	EtOH	Emran et al., 2012a
Anti-angiogenic	Leaves	EtOH	Avin et al., 2014
Antidiabetic	Leaves	EtOH, MeOH and water extracts	Ado et al., 2013; Dalu et al., 2014; Dalu and Dhulipala, 2016; Patel et al., 2016
Antidiarrhoeal	Leaves	MeOH extract	Tareq et al., 2017
Anti-inflammatory	Whole plant and leaves	MeOH extract and its *n*-hexane fraction	Saha et al., 2004; Sakib et al., 2021
Antimicrobial	Flowers, fruits, leaves, roots and stem	EtOH, MeOH, water and DCM extracts	A;I et al., 1996; Wiart et al. (2004); Srinivasan et al., 2009; Rahman et al., 2013a,b; Razak et al., 2014; Ramesh et al., 2015; Chander and Vijayachari, 2016, 2018;Harun et al., 2016; Rokhade and Taranath, 2016, 2017; Tareq et al., 2017; Mahboob et al., 2020
Antioxidant	Whole plant, leaves,	EtOH, MeOH, water extracts, and *n*-hexane, EtOAc and water fractions,	Saha et al., 2004; Emran et al., 2012a,b; Raihan et al., 2012; Reddy et al., 2012; Rahman et al., 2013a,b; Ramesh et al., 2015; Chander and Vijayachari, 2016; Ghahane et al., 2017; Sulistyaningsih et al., 2017; Ismail et al., 2019;
Antiproliferative	Leaves	EtOH, MeOH, water extracts, and n-hexane, EtOAc and water fractions	Nurhanan et al., 2008; Wong and Kadir, 2011, 2012; Emran et al., 2012a,b; Paul and Saha, 2012; Rahman 2013a,b; Raihan et al., 2012; Reddy et al., 2012; Ghagane et al., 2017; Siew at all., 2019
Effect on the central nervous system	Leaves	MeOH extract	Raihan et al., 2011; Sarris et al., 2013; Hosen et al., 2018; Chen et al., 2019
Hepatoprotective	Stem bark	EtOH extract	Mishra et al., 2014
Larvicidal	Leaves	MeOH extract	Sreedhanya et al., 2017
Phosphodiesterase inhibitory	Roots	EtOH extract	Temkitthawon et al., 2008, 2011
Thrombolytic	Leaves	EtOH, MeOH extracts and *n*-hexane fraction of MeOH extract	Rahman et al., 2013; Azad et al., 2018; Sakib et al., 2021
Wound healing	Aerial parts	EtOH extract	Wan et al., 2016

Antidiarrhoeal activity

The antidiarrhoeal activity of a methanolic extract of *L. indica* leaves was assessed by the castor oil-induced diarrhoea in mice (Tareq et al.2017). The extract at the doses of 500 mg/kg and 250 mg/kg significantly reduced the total number of stool as well as increased the latency period of defecation in comparison to the control groups. This appears to be the only antidiarrhoeal study reported on this plant to date.

Anti-inflammatory activity

A methanolic extract of *L. indica* whole plant exhibited strong inhibitory effect of nitric oxide (NO) production in lipopolysaccharide and interferon-γ induced mouse macrophage RAW 264.7 cells with percentage of NO inhibition 83.63, 80.42 and 74.91% at concentrations of 250, 125.5 and 62.5 µg/mL, respectively (Saha et al., 2004). Most recently, Sakib et al. (2021) reported that the *n*-hexane fraction of a methanol extract of *L. indica* leaves had a significant dose-dependent inhibition of haemolysis and protein denaturation compared to two non-steroidal anti-inflammatory drugs. This finding was in line with the traditional use of *L. indica* to relieve pain.

Antimicrobial activity

There are a good number of antimicrobial studies with *L. indica* extracts reported in the literature, several of which, however, are of preliminary levels, and based only on agar diffusion assays (Wiart et al., 2004; Chander and Vijayachari, 2016, 2018); some of them showed some antimicrobial activities, whereas the others did not. A methanolic extract of *L. indica* leaves was found inactive against Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Proteus mirabilis, Shigella flexneri* and *Salmonella typhi*) along with fungal strains (*Candida albicans* and *Aspergillus niger*) (Wiart et al., 2004; Chander and Vijayachari, 2016, 2018). However, a similar extract was reported to have antimicrobial activity against four Gram-positive pathogenic bacteria (*B. subtilis*, *B. cereus*, *B. megateriuum* and *S. aureus*), four Gram-negative pathogenic bacteria (*P. aeruginosa*, *S. dysenteriae*, *S. sonnei* and *Vibrio cholera*) and five fungal species (*A. niger*, *Blastomyces dermatitidis*, *C. albicans, Trichophyton spp., Microsporum spp.* and *Cryptococcus neoformans*) (Tareq et al., 2017). Similarly, methanolic extracts of the leaves and stem bark of *L. indica* displayed antifungal activity against fungal strains namely *Colletotrichum capsici*, *Helminthosporium sp*. and *Curvularia sp*. (Ramesh et al., 2015). Inconsistencies in the antimicrobial activities in different studies might be due to the differences in extraction techniques and assay protocols.

An ethanolic extract obtained from leaves of *L. indica* at three different concentrations 1, 2, and 3 mg/disk showed significant ($P < 0.05$) zones of inhibition (9.0-12.0 mm) against Gram-positive bacteria including *B. subtilis*, *S. aureus*, *B. cereus* and *B. megaterium* and Gram-negative bacteria namely *Salmonella typhi*, *Salmonella paratyphi*, *P. aeruginosa*, *Vibrio cholerae*, *Shigella dysenteriae* and *E. coli*, compared to that for the antibiotic tetracycline and ampicillin (16-20 mm) at 30 µg/disc (Rahman, Imran and Islam, 2013a,b). Moreover, this extract at 10 mg/disc inhibited the growth of *Aspergillus flavus*, *C. albicans* and *Fusarium equisetii* by 38.09 ± 0.59%, 22.58 ± 2.22% and 61.82 ± 2.7%, (fluconazole 67.01 ± 1.8%, 40.00 ± 2.5% and 72.32 ± 2.3%, respectively at 100 µg/disc) The minimum inhibitory concentrations (MIC) of the extract for different bacterial strains ranged from 25 to 100 µl/mL.

The essential oil derived from the flowers of *L. indica* demonstrated good antibacterial activity against two Gram-negative bacteria (*E. coli* and *S. typhimurium*), moderate activity against three Gram-positive bacteria (*B. subtilis*, *B. cereus*, and *S. aureus*), good antifungal activity against *Pencillium notatum* and moderate antifungal activity against two fungal strains (*A. niger* and *F. monelliforme*) (Srinivasan et al., 2009). The three largest zones of inhibition were observed with *P. notatum, S. typhimurium*, and *E. coli* (21, 11 and 10 mm, respectively). It was suggested that the antimicrobial effect of the essential oil might be due to the presence of phthalates in higher percentage. A dichloromethane (DCM) extracts of *L. indica* roots, stems and leaves exhibited antibacterial effect against *S. epidermis* and *S. aureus* (Harun et al., 2016); The DCM extract of the leaves at concentration 200 mg/mL produced the largest zone of inhibition (18 mm) against *S. epidermis*. Aqueous extracts of *L. indica* leaves and fruits were used for the synthesis of silver nanoparticles, which exhibited antimicrobial activity in combination of silver nanoparticles with antibiotic against *E. coli*, *S. typhi*, *S. aureus* and *B. subtilis* (Rokhade and Taranath, 2016; Rokhade and Taranath, 2017).

The only antiviral activity of *L. indica* was reported from an ethanolic extract of the leaves against *Herpes simplex* virus type-1 with an MIC value of 0.05 mg/mL (Ali et al., 1996). However, the extract was ineffective against *vesicular stomatitis* virus.

Antioxidant activity

A methanolic extract obtained from the whole plant of *L. indica* was analysed for antioxidant activity using the ferric thiocyanate, thiobarbituric acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging methods, and strong antioxidant activity was observed in all assays (Saha et al., 2004). Similarly, an ethanolic extract of *L. indica* leaves, and its *n*-hexane, ethyl acetate and water fractions were assessed for antioxidant property by the DPPH radical-scavenging, reducing power and superoxide dismutase (SOD) activity assays. Water fraction showed the strongest

DPPH radical-scavenging activity with an EC_{50} value of 48.0 µg/mL compared to that of ascorbic acid (AA) 15.0 µg/mL, a significantly ($p < 0.05$) higher reducing power with 2.70 ± 0.02 compared to AA 2.73 ± 0.03 at 0.8 mg/mL and the strongest inhibition rate ($p < 0.05$) in the SOD assay which could be attributed by the high content of phenolic compounds in water fraction (Reddy et al., 2012). Similar antioxidant activity of an ethanolic extract was also reported by Rahman et al., (2013a,b), where a strong DPPH-radical scavenging activity (IC_{50} = 139.83 ± 1.40 µg/mL compared to AA IC_{50} 1.46 ±0.06 µg/mL) was observed. Additionally, the ethanolic extract displayed $FeCl_3$ reduction with IC_{50} = 16.48 ± 0.64 µg/mL compared to AA IC_{50} = 14.04 ± 1.20 µg/mL and superoxide radical scavenging effect 49.54 ± 0.51% with IC_{50} = 676.08 ± 5.80 µg/mL, compared to curcumin with IC_{50} = 60.48 ± 0.53%, along with less potent iron chelating activity with IC_{50} = 519.33 ± 16.96 µg/mL compared to AA IC_{50} = 8.81 ± 0.90 µg/mL.

Ghagane et al. (2017) reported that the methanolic extract of the leaves of *L. indica* had higher antioxidant activity than the ethanolic and aqueous extracts in the DPPH, ferric ion reducing power and phosphomolybdenum assays. The aqueous and methanolic extracts of the leaves showed prominent effects in the DPPH assay with the IC_{50} values of 0.27 and 0.28 mg/mL, respectively, using gallic acid as the control (IC_{50} = 0.28 mg/mL) (Ismail et al., 2019). There are several other similar DPPH assay-based antioxidant activity assessments of *L. indica* reported in the literature (Emran et al., 2012; Raihan et al., 2012; Ramesh et al., 2015; Chander and Vijayachari, 2016; Sulistyaningsih et al, 2017 and Chen et al., 2019). Different research suggested that the antioxidant potential of *L. indica* could be due to the presence of phenolic compounds as well as some alkaloids, terpenoids, sterols, saponins (Emran et al., 2012; Reddy et al., 2012; Ghagane et al.,2017; Chen et al., 2019). It is well-established that phenolic compounds possess antioxidant activity due to their redox properties which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Liang et al., 2010 and Luna-Guevara et al., 2018).

Antiproliferative activity

In vitro assessment of antiproliferative activity of plant extracts against cancer and tumour cell lines, often in the MTT or sulforhodamine-B assays, generally provides indications about their potential anticancer and antitumour activities. In a sulforhodamine-B assay, conducted by Nurhanan et al. (2018), using methanol extracts of the leaves and stem bark of *L. indica*, but no noticeable antiproliferative activity was observed against the MCF-7 and T47D breast cancer cell lines (IC_{50} > 100 µg/mL in both cell lines). Similar inactivity was observed in several other studies using the MTT assay. A crude ethanol extract and its fractions (ethyl acetate, *n*-hexane and water) were tested against Ca Ski, MCF-7, MDA-MB-435, KB, HEP G2, WRL 68 and Vero cell lines by the MTT assay, and except for the activity of ethyl acetate (EtOAc) fraction of the ethanol (EtOH) extract against Ca Ski cervical cancer cells (IC_{50} = 85.83 ± 6.01 µg/mL), none of the other fractions or extracts demonstrated significant activity against any of the cell lines as demonstrated by their IC_{50} values of >180 µg/mL. However, the EtOAc fraction was found to decrease cell viability of MCF-7, KB, MDA-MB-435, KB, HEP G2 and WRL 68 cells, and to induce apoptosis via nuclear shrinkage, chromatin condensation, increase in sub-G1 cells, DNA fragmentation, intracellular GSH depletion and caspase-3 activation (Wong and Kadir, 2011). Extracts and fractions were evaluated against three human colon cancer cell lines, HT-29, HCT-15 and HCT-116, but no antiproliferative activity could be observed (Reddy et al., 2012). Similarly, an EtOH extract of the leaves of *L. indica* revealed no significant antiproliferative activity against Vero cells (Wong and Kadir, 2011) and HeLa cells (Ali et al.,1996). Nonetheless, a moderate cytotoxic activity was observed against MCF-7 cell line (Azad et al., 2018).

Ghagane et al. (2017), using the MTT assay, showed *in vitro* cytotoxicity of three different extracts (EtOH, MeOH and water) of the leaves of *L. indica* against two human prostate cancer cell lines, DU-145 and PC-3, while no cytotoxicity was observed on normal mice embryo fibroblast cell line (MEF-L929). Among the extracts, the methanolic extract inhibited human prostate cancer cell lines DU-145 and PC-3 with IC_{50} values of 529.44 ± 42.07 µg/mL and 547.55 ± 33.52 µg/mL, respectively. The IC_{50} values, although the authors claimed cytotoxicity, could not establish that the extracts were really cytotoxic, as the IC_{50} values were well above 100 µg/mL, which would not usually be considered as cytotoxic for any anticancer drug development purposes.

In a recent study, the antiproliferative activity of seven medicinal plants including *L. indica* leaves using water soluble tetrazolium salt (WST-1) assay was evaluated on twelve human cancer cell lines derived from breast (MDA-MB-231, T47D), cervical (C33A), colon (HCT116), leukaemia (U937), liver (HepG2, SNU-182, SNU-449), ovarian (OVCAR-5, PA-1, SK-OV-3) and uterine (MES-SA/DX5) cancer. All leaf extracts of *L. indica* demonstrated strong or moderately strong antiproliferative activity against almost all cell lines tested. However, not all leaf extracts were active against leukaemic U937 cells. The most effective one was the methanolic extract of *L. indica* with the IC_{50}

values of 31.5 ± 11.4 µg/mL, 37.5 ± 0.7 µg/mL and 43.0 ± 6.2 µg/mL in cervical C33A, liver SNU-449, and ovarian PA-1 cancer cell lines, respectively (Siew at all., 2019).

An ethanolic extract of the leaves of *L. indica* displayed brine shrimp toxicity (not really cytotoxicity as described by the authors) at lethal concentrations (LC_{50}) of 2.48 µg/mL (Emran et al., 2012; Paul and Saha, 2012), 2.65 ± 0.16 µg/mL (Rahman et al., 2013a, 2013b) and 170.86 µg/mL (Azad et al., 2018). However, brine shrimp lethality assay, although not an assay that could provide any cytotoxicity data, is often incorrectly used to assess cytotoxicity of plant extracts and compounds. This assay merely shows the lethality against brine shrimp, which can be attributed by various factors, but not necessarily only because of cytotoxicity.

In vivo antitumour activity of a methanolic extract of the *L. indica* leaves was assessed against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice. The results demonstrated that the extract at a dose of 40 mg/kg, i.p. displayed maximum antitumour activity with 77.29% of cell growth inhibition compared to the positive control bleomycin with 92.02% of cell growth inhibition (0.3 mg/kg, i.p.). A reduction in the tumour weight (7.90 g) was also observed as was an enhancement of the life span by 69.33%. The positive control bleomycin at a dose of 0.3 mg/kg (i.p.) showed 7.05 g of tumour weight reduction and 94.66% ($p < 0.01$) increase in life span (Raihan et al., 2012).

Central nervous system (CNS)- affecting activity

A methanolic extract of the leaves of *L. indica* showed CNS-affecting properties as evidenced from its sedative and anxiolytic (Raihan et al., 2011) and anti-amnesic effects (Chen et al., 2019). Administrations of the extract at the doses of 200 and 400 mg/kg displayed dose-dependent anxiolytic effects (in hole cross and open-field tests), and suppression of motor activity and prolongation of thiopental-induced sleeping time. As *L. indica* leaves contain ursolic acid, eicosanol, farnesol and β-sitosterol which are γ-aminobutyric acid type A (GABAA) agonists, those phytochemicals could be responsible for the above CNS-depressant effects of this plant (Raihan et al., 2011). The extract at 500 mg/kg, p.o., exerted anti-amnesic effect on scopolamine-induced amnesia of Alzheimer's type in rats and significantly modulated the induced memory deficits, which could be related to its anti-acetylcholinesterase, antioxidant and anti-inflammatory activities (Chen et al., 2019). Combined molecular docking and molecular dynamics-based techniques were used to find a potent inhibitor of BACE1 (beta secretase 1) from the components derived from *L. indica*, and this study explored lupeol as a potential lead molecule for a new therapeutic agent for Alzheimer's disease (Hosen et al., 2018). However, the conclusion was somewhat premature as appropriate *in vitro* and *in vivo* studies are necessary before this *in silico* study-based assumption could be substantiated.

Sarris et al. (2013) reviewed plant-based medicines for anxiety disorders on the basis of preclinical trials and found that *L. indica* could be useful to treat this disorder. It was suggested that the plausible mechanism of action could involve GABA either via direct receptor binding or ionic channel or cell membrane modulation.

Hepatoprotective activity

Generally, plants that contain high amounts of antioxidant compounds tend to offer hepatoprotective activity through mitigating damages caused by oxidative stress. An ethanolic extract of the stem bark of *L. indica* displayed hepatoprotective activity against paracetamol-induced hepatotoxicity *in vivo* in rats. The treatment of mice with the extract at two doses (200 mg/kg and 400 mg/kg body weight) could significantly reduce the elevated levels of serum marker enzymes, bilirubin and triglycerides, when compared to the positive control group (Mishra et al., 2014). As this plant is known to produce antioxidant phytochemicals, it was inferred that those antioxidants could be responsible for the hepatoprotective effect observed in the study.

Larvicidal activity

Larvicidal activity is desirable for pest (insect) control, a good example of which is the larvicidal activity of a methanolic extract of the leaves of *L. indica* was against *Culex quinquefasciatus* mosquitos (Sreedhanya et al., 2017).

Phosphodiesterase (PDE) inhibitory activity

An ethanolic extract of the roots of *L. indica* was screened for its PDE inhibitory activity using a radioactive-assay (Temkitthawon et al., 2008). The extract at a concentration of 0.1 mg/mL showed high PDE-inhibitory effect with an IC_{50} value of 2.62 ± 0.25 µg/mL, compared to that of the known PDE inhibitor 3-isobutyl-1-methylxanthine (IC_{50} 0.68 ± 0.14 µg/mL). In addition, this extract displayed a 31.36 ± 7.47% PDE-5 inhibition in the two-step radioactive assay (Temkitthawon et al., 2011).

Thrombolytic activity

Potential thrombolytic property of an ethanolic extract of the leaves of *L. indica* was assessed as the clot-lysis effect (39.3 ± 0.96%) and compared with the effect offered by the positive control drug streptokinase (Rahman et al., 2013). Later, Sakib et al. (2021) demonstrated that the *n*-hexane fraction from the MeOH extract could exhibit significant thrombolytic activity (32.58 ± 1.18%). Previously, however, another study revealed a moderate level of clot-lysis activity of an ethanolic extract (07.24 ± 0.15%) (Azad et al., 2018). It was suggested that the observed thrombolytic effects might be linked to the antibacterial activity of this plant (Rahman et al., 2013).

Wound healing activity

An ethanolic extract of the aerial parts of *L. indica* was found to possess diabetic wound healing property, as revealed from the scratch assay using NIH 3T3 mouse fibroblast and Raw 264.7 mouse macrophage cells (Wan et al., 2012). The extract could enhance the migration of the cells towards the closure of the gap, and thus heal the wound. It was assumed that the activity might be associated with high antioxidant activity offered by tannins present in the extract.

Various other activities

An *n*-hexane fraction of a methanolic extract of the leaves of *L. indica* leaves exhibited hair growth-promoting activity (Sakib et al. 2021). When applied on the mice skin at the concentrations of 10, 1, 0.1%, it demonstrated a significant increase in average hair length ($p < 0.001$) compared with untreated animals. This fraction at a concentration of 1% exhibited the highest percentage of hair regrowth on day 7, 14 and 21 (81.24, 65.60, and 62.5%, respectively). The DCM, MeOH and water extracts of *L. indica* leaves were tested for antiplasmodial activity against chloroquine resistant *Plasmodium falciparum* by using HRP2 assay, but the activities were weak or inactive (EC_{50} > 15.7 µg/mL) (Mohammadd Abdur Razak et al., 2014). Nonetheless, the methanolic extract of *L. indica* leaves demonstrated antimalarial activity in a malaria-induced mice model (the 4-day suppressive test) (Sulistyaningsiha et al., 2017). Mahboob et al. (2020) reported amoebicidal activity of the water and butanol fractions of the EtOH extract of the leaves against trophozoites and cysts.

Therapeutic potential isolated compounds

Most of the studies aiming at assessing the therapeutic potential of *L. indica* were confined to preliminary *in vitro*, and some *in vivo* animal assays with crude extracts and their solvent fractions, without any major efforts in conducting bioassay-guided isolation of active therapeutic agents from this plant. Phytochemical studies were mainly qualitative phytochemical screening for detecting the presence of certain groups of phytochemicals, with some GC-MS and LC-MS analyses of active extracts/fraction tentatively identifying the presence of some phytochemicals as discussed in the previous sections. Only a handful of reports are available on proper isolation and identification of active compounds from this plant. Also, there could hardly any attempts be observed to assess the therapeutic potential of purified compounds from active extracts/fractions.

One of the major bioactivity studies with isolated compounds from *L. indica* was conducted by Wong's group (Wong et al., 2012; Wong and Kader, 2012). Two cycloartane triterpenoid glycosides, mollic acid α-L-arabinoside and mollic acid β-D-xyloside (Figure 7) were isolated from the active EtOAc fraction of *L. indica* leaves. Both identified compounds inhibited the growth of Ca Ski cells with IC_{50} value of 19.21 and 33.33 µM, respectively. Compared to the MRC5 cell line, both terpenoids were between 4-8 fold more cytotoxic, respectively, to Ca Ski cells. The cytotoxicity of mollic acid α-L-arabinoside was associated with a decrease in proliferating cell nuclear antigen gene expression, cell cycle arrest at S and G2/M phases, as well as induction of hypodiploid cells (Wong, Kadir and Ling, 2012). This compound induced mitochondrial-mediated apoptosis in Ca Ski cells by promoting upregulation of Bax and downregulation of Bcl-2 (Wong and Kadir, 2012). It is interesting to note that despite the extracts and fractions of *L. indica* not showing any significant antiproliferative activity (IC_{50} = >100 µg/mL), these isolated triterpenes from weakly active EtOAc fraction of the methanolic extract showed quite prominent cytotoxicity against certain cell lines. Several other triterpenes (Figure 8) reported from *L. indica*, *e.g.*, α-amyrin, lupeol and ursolic acid, are well-known for their bioactivities including antiproliferative activity (Nahar and Sarker, 2019), and thus, their presence in *L. indica* might provide some rationale behind the traditional use of this plant in the treatment some forms of cancers and tumours. However, much more work is needed, especially structured pre-clinical and clinical trials before the true therapeutic efficacy of *L. indica* extracts and their major bioactive compounds can be established conclusively.

L. indica has been shown to possess several phenolic and polyphenolic compounds including dihydrochalcones, flavonoids and tannins, which are generally well-known for significant antioxidant property (Nahar and Sarker, 2019).

Although most of these compounds were identified by LC-MS data analyses, some of those compounds, *e.g.,* ellagic acid, gallic acid and quercetin, were purified and tested for *in vitro* antioxidant activities. However, some non-phenolic compounds were also isolated as the major contributors for the antioxidant property of the extracts of *L. indica*. For example, a recent investigation of the tentative antioxidative constituents from stem and leaves extracts of *L. indica* revealed the presence of 1-(hydroxymethyl)-1,2-ethanediyl ester (LI-1), 9-oxononanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester and 3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone as antioxidant components (Baharom et al., 2020).

Antiamoebic activity of *L. indica* was attributed to gallic acid. Gallic acid encapsulated in the PLGA nanoparticles exhibited 90% inhibition against trophozoites and reduced cytotoxicity towards MRC-5 compared to gallic acid. In the assessment of antidiabetic potential of this plant, it was suggested that the antihyperglycemic and hypolipidemic properties of *L. indica* could be due to the presence of ursolic acid and gallic acid, as ursolic acid was reported as an effective insulin-mimetic agent and gallic acid was reported to be an insulin-secretagogue, antihyperlipidemic and antioxidant (Dalu et al., 2014 and Dalu and Dhulipala, 2016). Again, there is no report on any bioassay-guided isolation of antidiabetic compounds and subsequent assessment for antidiabetic therapeutic potential of those compounds. However, as one of the mechanisms for antidiabetic actions is directly linked to reduction of oxidative stress, the presence of high amounts of antioxidant compounds in *L. indica*, could be a major contributor for its antidiabetic activity.

In an *in silico* study, two triterpenoids, ursolic acid and lupeol, isolated from *L. indica*, were identified as potent BACE1 inhibitor from a manually curated dataset of *L. indica* molecules, which might offer a novel direction for designing novel BACE1 inhibitors as therapeutic options for Alzheimer disease (Hosen et al., 2018).

CONCLUSIONS

Preliminary in vitro and some in vivo studies involving animal models with the extracts and fractions of L. indica provided some scientific basis for traditional therapeutic applications of this plant. The presence of certain bioactive compounds in the extracts and fractions could further support therapeutic potential and traditional medicinal uses of this plant. However, more extensive phytochemical work leading to isolation of active compounds, and subsequent assessment of their therapeutic efficacy in relation to certain disease conditions is still needed. Although this plant is still in use in traditional medicinal practices in Asia, and present in some of the traditional medicinal preparations, well-designed pre-clinical and clinical studies are essential before any recommendations could be made on the efficacy and safety of L. indica-based therapeutic interventions.

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CONFLICT INTEREST

The author declares no personal or financial conflict of interest related to this work.

AUTHORS CONTRIBUTION

(C.K.) (L.N.) (K.J.R.) (S.D.S.) Conceptualization, Methodology, Formal analysis, Investigation, Writing, Review & Editing.

REFERENCES

Ado MA, Abas F, Mohammed AS and Ghazali HM (2013). Anti- and pro-lipase activity of selected medicinal, herbal and aquatic plants, and structure elucidation of an anti-lipase compound. *Molecules* 18, 14651-14669.

Ali AM, Mackeen MMM, El-Sharkawy SH, Hamid J, Ismail N, Ahmad FBH and Lajis NH (1996). Antiviral and cytotoxic activities of some plants used in Malaysian indigenous medicine. *Pertanika Journal of Tropical Agricultural Science* 19, 129-136.

Avin BRV, Thirusangu P, Ramesh CK, Vigneshwaran V, Kumar MVP, Mahmood R and Prabhakar BT (2014). Screening for the modulation of neovessel formation in non-tumorigenic and tumorigenic conditions using three different plants native to Western ghats of India. *Biomedicine and Aging Pathology* 4, 343-348.

Azad AK, Jainul MA and Labu ZK (2018). Cytotoxic activity on brine shrimp, MCF-7 cell line and thrombolytic potential: Seven different medicinal plant leaves extract. *Journal of Scientific Research* 10, 175-185.

Baharom FA, Mohd Azam NN, Daud S, Mat So'ad SZ, Mohd Hassan N and Harun A (2020). Identification of tentative antioxidant constituents from stem and leaves of *Leea indica* species using spectroscopic analysis. *GADING Journal of Science and Technology* 3, 129-136.

Chander MP and Vijayachari P (2016). *In vitro* antimicrobial and anti-oxidant potentials of selected medicinal plants used by the indigenous tribes of Andaman and Nicobar Islands, India. *Bangladesh Journal of Pharmacology* 11, 330-332.

Chander MP and Vijayachari P (2018). Antimicrobial Properties of Ethnomedicinal Plants against Selected Human Pathogens. *Journal of Traditional Medicine and Clinical Naturopathy* 7, article number: 253.

Chen Q, Chen X, Fu Z, Bais S and Hou X (2019) Anti-amnesic effect of *Leea indica* extract in scopolamine-induced amnesia of Alzheimer's type in rats. *International Journal of Pharmacology* 15, 116-123.

Dalu D and Dhulipala S (2016). Evaluation of possible mechanisms of three plants for blood glucose control in diabetes. *Bangladesh Journal of Pharmacology* 11, 224-230.

Dalu D, Duggirala S and Akarapu S (2014). Anti-hyperglycemic and hypolipidemic activity of *Leea indica*. *International Journal of Bioassays* 3, 3155-3159.

Emran TB, Rahman MA, Hosen SMZ, Rahman MM, Islam AMT, Chowdhury MAU and Uddin ME (2012a). Analgesic activity of *Leea indica* (Burm. f.) Merr. *Phytopharmacology* 3, 150-157.

Emran TB, Rahman MA, Hosen ZS, Khanam UH and Saha D (2012b). Antioxidant, cytotoxic and phytochemical properties of the ethanol extract of *Leea indica* leaf. *Journal of Pharmaceutical Research* 5, 2938-2941.

Ghagane SC, Puranik SI, Kumbar VM, Nerli RB, Jalalpure SS, Hiremath MB, Neelagund S and Aladakatti R (2017). *In vitro* antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. *Integrative Medicine Research* 6, 79-87.

Harun A, Ab Rahim NES, Jalil MAA, Rosdi NAM, Daud S, Harith SS, So'ad SZM and Hassan NM (2016). Comparative study of antioxidant and antimicrobial activity of root, stem and leaves of *Leea indica* species. *Malaysian Journal of Science* 35, 259-274.

Harun A, Daud S, Harith SS, Rahim AA, Jalil MA, Rosdi NAM, Azieyati F, Nabihah N, So'ad SZM and Hassan NM (2018). Radical scavenging activity and phytochemical investigation of antioxidative compounds from stem and leaves *of Leea Indica* species. *Journal for Science and Technology* 1, 52-59.

Hosen SMZ, Rubayed M, Dash R, Junaid M, Mitra S, Alam MS and Dey R (2018). Prospecting and structural snsight into the binding of novel plant-derived molecules of *Leea indica* as inhibitors of BACE1. *Current Pharmaceutical Design* 24, 3972-3979.

Ismail A, Mohamed M, Kwei YF and Yin KB (2019). *Euphorbia hirta* methanolic extract displays potential antioxidant activity for the development of local natural products. *Pharmacognosy Research* 11, 78-85.

Joshi A, Tari P and Bhobe M (2013). Phytochemical investigation of the roots of *Leea indica* (Burm. F.) Merr. *International Journal of Research in Pharmaceutical and Biomedical Sciences* 4, 919-925.

Kekuda TRP, Raghavendra HL, Bharadwaj NA and Akhilesha S (2018). Traditional uses, chemistry and pharmacological activities of *Leea indica* (Burm. f.) Merr. (Vitaceae): a comprehensive review. *International Journal of Green Pharmacy* 12, 71-80.

Liang T, Yue W and Li Q (2010). Comparison of the phenolic content and antioxidant activities of *Apocynum venetum* L. (Luo-Bu-Ma) and two of its alternative species. *International Journal of Molecular Sciences* 11, 4452-4464.

Luna-Guevara ML, Luna-Guevara JJ, Hernández-Carranza P, Ruíz-Espinosa H and Ochoa-Velasco CE (2018). Phenolic compounds: A good choice against chronic degenerative diseases. In: Atta ur, R. (ed.) *Studies in Natural Products Chemistry*. Elsevier, pp. 79-108.

Mishra G, Khosa R, Singh P and Jha K (2014). Hepatoprotective activity of ethanolic extract of *Leea indica* (Burm.f.) Merr. (Leeaceae) stem bark against paracetamol induced liver toxicity in rats. *Nigerian Journal of Experimental and Clinical Biosciences* 2, 59-63.

Mishra G, Khosa R, Singh P and Jha K (2016). Ethnobotany and pharmacology of *Leea indica* (Burm.f.). *Journal of Costal Life Medicine* 4, 69-72.

Mohd Abd Razak MR, Afzan A, Ali R, Amir Jalaluddin NF, Wasiman MI, Shiekh Zahari SH, Abdullah NR and Ismail Z (2014). Effect of selected local medicinal plants on the asexual blood stage of chloroquine resistant *Plasmodium falciparum*. *BMC Complementary and Alternative Medicine* 14, article number: 492.

Mollik AH, Hasan N, Hossan S, Johan R and Rahmatullah M (2009). Medicinal plants used against leucorrhea in several regions of Bogra district, Bangladesh. *Planta Medica* 75, 954-954.

Nahar L and Sarker SD (2019). Chemistry for Pharmacy Students: General, Organic and Natural Product Chemistry, 2nd edition, Wiley and Sons, Chichester, UK.

Nurhanan MY, Asiah O, Ilham MAM, Syarifah MMS, Norhayati I and Sahira HL (2008). Anti-proliferative activities of 32 Malaysian plant species in breast cancer cell lines. *Journal of Tropical Forest Science* 20, 77-81.

Patel B, Patel J and Shah S (2016). Effect of single and combinational herbal formulation in alloxan induced hyperglycemia. *Der Pharmacia Lettre* 8, 114-120.

Patel AA, Amin AA, Patwari AH and Shah MB (2017). Validated high performance thin layer chromatography method for simultaneous determination of quercetin and gallic acid in *Leea indica*. *Revista Brasileira de Farmacognosia* 27, 50-53.

Paul S and Saha D (2012). Cytotoxic activity of ethanol extract of *Leea indica* leaf. *Asian Journal of Research in Pharmaceutical Science* 2, 137-139.

Rahman MA, Imran TB and Islam S. (2013a). Antioxidative, antimicrobial and cytotoxic effects of the phenolics of *Leea indica* leaf extract. *Saudi Journal of Biological Science* 20, 213-225.

Rahman MA, Sultana R, Bin Emran T, Islam MS, Rahman MA, Chakma JS, Rashid HU and Hasan CM (2013b). Effects of organic extracts of six Bangladeshi plants on *in vitro* thrombolysis and cytotoxicity. *BMC Complementary and Alternative Medicine* 13, article number: 25.

Raihan MO, Habib MR, Brishti A, Rahman MM, Saleheen MM and Manna M (2011). Sedative and anxiolytic effects of the methanolic extract of *Leea indica* (Burm. f.) *Merr. leaf. Drug Discovery and Therapeutics* 5, 185-189.

Raihan MO, Tareq SM, Brishti A, Alam MK, Haque A and Ali MS (2012). Evaluation of antitumor activity of *Leea indica* (Burm.f.) Merr. extract against Ehrlich Ascites Carcinoma (EAC) bearing mice. *American Journal of Biomedical Sciences* 4, 143-152.

Ramesh D, Ramesh D, Prashith KTR, Onkarappa R, Vinayaka KS and Raghavendra HL (2015). Antifungal and radical scavenging activity of leaf and bark of *Leea indica* (Burm. f.) Merr. *Journal of Chemical and Pharmaceutical Research* 7, 105-110.

Reddy NS, Navanesan S, Sinniah SK, Wahab NA and Sim KS (2012). Phenolic content, antioxidant effect and cytotoxic activity of *Leea indica* leaves. *BMC Complementary and Alternative Medicine* 12, article number: 128.

Rokhade VK and Taranath TC (2016). Biosynthesis of silver nanoparticles using leaf extract of *Leea Indica* (Burm. F.) Merr. and their synergistic antimicrobial activity with antibiotics. *International Journal of Pharmaceutical Sciences Review and Research* 40, 211-217.

Rokhade VK and Taranath TC (2017). Phytosynthesis of silver nanoparticles using fruit extract of *Leea indica* (Burm. F.) Merr. and their synergistic antimicrobial activity. *International Journal of Pharmaceutical Sciences and Research* 8, 1319-1325.

Saha K, Lajis NH, Israf DA, Hamza, AS, Khozirah S, Khamis . and Syahida A (2004). Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *Journal of Ethnopharmacology* 92, 263-267.

Sakib SA, Tareq AM, Islam A, Rakib A, Islam MN, Uddin MA, Rahman MM, Seidel V and Emran TB (2021). Anti-Inflammatory, thrombolytic and hair-growth promoting activity of the *n*-hexane fraction of the methanol extract of *Leea indica* leaves. *Plants* 10, article number: 1081.

Sarris J, McIntyre E and Cranfield DA (2013). Plant-based medicines for anxiety disorders, Part 1. A review of preclinical studies. *CNS Drugs* 37, 207-219.

Siew YY, Yew HC, Neo SY, Seow SV, Lew SM, Lim SW, Lim CSES, Ng YC, Seetoh WG and Ali A (2019). Evaluation of anti-proliferative activity of medicinal plants used in Asian traditional medicine to treat cancer. *Journal of Ethnopharmacology* 235, 75-87.

Singh D, Siew YY, Chong TI, Yew HC, Ho SS, Lim CSE, Tan WX, Neo SY and Koh HL (2019a). Identification of phytoconstituents in *Leea indica* (Burm. f.) Merr. leaves by high performance liquid chromatography micro time-of-flight mass spectrometry. *Molecules* 24, article number: 714.

Singh D, Siew YY, Yew HC, Neo SY and Koh HL (2019b). Botany, phytochemistry and pharmacological activities of *Leea* species. In: Swamy MK, Patra JK and Rudramurthy GR (ed.) *Medicinal Plants: Chemistry, Pharmacology, and Therapeutic Applications*. Florida: CRC Press. pp. 11-41.

Sreedhanya S, Athira A and Pushpalatha E (2017). Larvicidal and repellent efficacy of some of the weed plant extracts against *Culex Quinquefasciatus* Say. *Journal of Advanced Laboratory Research in Biology* 8, 6-11.

Srinivasan GV, Ranjith C and Vijayan KK (2008). Identification of chemical compounds from the leaves of *Leea indica*. *Acta Pharmaceutica* 58, 207-214.

Srinivasan GV, Sharanappa P, Leela N, Sadashiva CT and Vijayan K. (2009). Chemical composition and antimicrobial activity of the essential oil of *Leea indica* (Burm. f.) Merr. flowers. *Natural Product Radiance* 8, 488-493.

Sulistyaningsiha E, Amalia TY and Kartikasari R (2017) Antioxidant and antimalarial activity of *Leea indica* leaf extract against malaria-mice model. *Journal of Applied Pharmaceutical Science* 7, 163-168.

Tareq SM, Ibrahim M, Shahadat S, Chowdhury MU and Jakaria M (2017). Comparative anti-dirrhoeal and antimicrobial activities of methanol extract of *Leea indica* (Burm. F.) Merr. and *Leea macrophylla* Roxb. Ex. Hornem (Fam. Vitaceae) and four Bangladeshi market preparations. *Der Pharmaceutical Chemistry* 9, 27-34.

Temkitthawon P, Viyoch J, Limpeanchob N, Pongamornkul W, Sirikul C, Kumpila A, Suwanborirux K and Ingkaninan K (2008). Screening for phosphodiesterase inhibitory activity of Thai medicinal plants. *Journal of Ethnopharmacology* 119, 214-217.

Temkitthawon P, Hinds TR, Beavo JA, Viyoch J, Suwanborirux K, Pongamornkul W, Sawasdee P and Ingkaninan K (2011). *Kaempferia parviflora*, a plant used in traditional medicine to enhance sexual performance contains large amounts of low affinity PDE5 inhibitors. *Journal of Ethnopharmacology* 137, 1437-1441.

Wan MAWS, Sunzida NK. and Azad AK (2016). The screening of local herbs in treating non healing wounds and diabetic foot ulcers complications using nih 3t3 mouse fibroblast and raw 264.7 mouse macrophage cells. *Pharmacology Online*, 1, 139-145.

Wiart C, Mogana S, Khalifah S, Mahan M, Ismail S, Buckle M, Narayana AK and Sulaiman M (2004). Antimicrobial screening of plants used for traditional medicine in the state of Perak, Peninsular Malaysia. *Fitoterapia* 75, 68-73.

Wong YH and Kadir HA (2011). *Leea indica* ethyl acetate fraction induces growth-inhibitory effect in various cancer cell lines and apoptosis in Ca Ski human cervical epidermid carcinoma cells. *Evidenced-based Complementary and Alternative Medicine* 2011, article number: 293060.

Wong YH and Kadir HA (2012). Induction of mitochondria-mediated apoptosis in Ca Ski human cervical cancer cells triggered by mollic acid arabinoside isolated from *Leea indica*. *Evidence-based Complementary and Alternative Medicine* 2012, article number: 684740.

Wong YH, Kadir HA and Ling SK (2012). Bioassay-guided isolation of cytotoxic cycloartane triterpenoid glycosides from the traditionally used medicinal plant *Leea indica*. *Evidence-based Complementary and Alternative Medicine* 2012, article number: 164689.

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Review

ANTIOXIDANT ACTIVITY OF *APIS MELLIFERA* BEE PROPOLIS: A REVIEW

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Background

Propolis is a natural product manufactured by bees from balsamic materials collected from plants that surround the hive, undergoing subsequent modification by the enzymes of these insects. It has several functions in the hive, such as sealing cracks and antimicrobial action. Folk medicine worldwide has used this resin in their health practices, and modern research turns its eyes to natural materials to become sources of new molecules to treat the most diverse ailments.

Aims

This work collected information on studies that test the antioxidant activity of propolis, produced by *Apis mellifera* bees, using different antioxidant methods available.

Methods

The search for this review was carried out in the following databases: SciELO, Google Scholar, PubMed, MEDLINE, Catalog of Dissertations and Theses of CAPES, BVS, CRD, Embase, Science Direct, Scopus and Cochrane Library. Publications in Portuguese, English and Spanish in the last decade were included.

Results

The 173 articles chosen showed quantitative and qualitative data about the potential of this natural product in the area of interest. Propolis extracts reached amazing values in antioxidant tests; they were as active as isolated substances already recognized as standard patterns. Many studies have brought information about the antioxidant mechanisms of propolis, such as free radical scavenging, metal chelation, and electron donation.

Conclusion

This review brings scientific evidence, *in vitro* and *in vivo*, that supports the idea that propolis is a good candidate for producing new antioxidant pharmaceutical and food formulations in the future.

Keywords: *Apis mellifera*; propolis; antioxidant activity

INTRODUCTION

Propolis is a complex set of balsamic substances arising from resins from different parts of plants (sprouts, flowers, branches, latex, bark) and plant exudates from lesions (1–3) collected by worker bees of the species *Apis mellifera* and stingless bees (Meliponini) (4,5). Although it is an animal product, most of the components of propolis, especially the active ones, come from plants (6). Bees can add pollen, wax, salivary enzymes, among others, increasing their biological activity (2,4,7,8). As it is a lipophilic compound, propolis has a hard and fragile appearance at room temperature, but it is sticky and elastic at higher temperatures (9). Due to its sticky and adhesive characteristics – owing to its great interaction with oils and skin proteins – (10), the expression “bee glue” emerged (11). It has a characteristic odor, bitter and astringent taste. The color varies, from brown to green, passing through red tones, according to its origin; however, dark brown is most common. The color of propolis is closely linked to its commercial value and quality parameters, with green (southeast Brazil) and red (northeast Brazil) being especially valued (6,12).

In hives, propolis plays an important role in covering cracks (1,11), building (10), embalming invaders' bodies (9), promoting thermal insulation (1,4), and, above all, it blocks microbial growth in essential areas of the hive (3). It reflects the origin of its name: from the Greek pro-, in defense, and polis-, city or community, that is, in defense of the community (hive) (1,11).

Although several bee species are capable of producing propolis, *A. mellifera* is recognized as the main producer among all of them. Annually, from each *A. mellifera* hive, 100 to 300 grams of propolis can be extracted, which makes this species a very efficient producer (4,13). Another important point regarding propolis is its high commercial value, with immense appreciation: in 2010, a kilo of propolis cost U$S 84.87, jumping to U$S 129.47 in two years (1). In some cities, such as Tokyo, Japan, the bottle can reach up to US$150, and one gram of propolis produced in Minas Gerais (Brazil) costs around US$200 (14). The propolis market is estimated to grow around USD 40 million from 2020 to 2025, with a CAGR (Compound Annual Growth Rate) of 5,41% (15).

Humankind has used this balsamic product since antiquity, with notes of applying this resin from Mesopotamia, ancient Greece, and Rome (1,16). In the second half of the 20th century, the use of propolis in medicine was disseminated around the world and adopted in integrative and complementary health practices and the scope of production of supplements and foods (17). The rich chemical composition of propolis explained its wide use. This natural product has fixed and volatile portions - some authors claim it has about 500 compounds (18). About 3% of them have some biological activity, which is ordinarily obtained through the synergistic interaction between several substances (19). The most widely known active molecules are phenolic compounds such as flavonoids and phenolic acids (1).

There is evidence of its antimicrobial action against several species of Gram-negative and Gram-positive bacteria, yeasts, and fungi, as well as an anticarcinogenic, anti-inflammatory, antioxidant, anesthetic (3,20), immunostimulant (21), anti-protozoal (1), healing (20), and antimutagenic functions (22). Recent studies have demonstrated the effectiveness of propolis in fighting SARSCoV-2, emphasizing the current importance of this product (23).

Among all biological activities that natural products have, the antioxidant stands out. This fact is due to the impact that oxidizing species, such as free radicals, have on various health problems, like aging, cell degeneration (24), cancer (3,25), immune system dysfunction, arthritis, diabetes, liver and kidney problems, Parkinson, and Alzheimer (4,11).

Antioxidant substances, synthetic or natural, are those present in the medium, even in small quantities, with the power to inhibit the oxidation of substrates (14,22). The mechanism of action of antioxidant substances is based on the inhibition of free radicals and interaction with their biological targets through the donation of electrons or hydrogens (primary antioxidants). Inhibition reactions produce stable, non-reactive molecules, which can later be degraded through other enzymes and processes. They can even act as chelators, scavenge transition metals, as they are generally catalysts for oxidative reactions, absorb ultraviolet radiation (secondary antioxidants), and scavenge oxygen (9,26,27).

For a molecule to be considered a good antioxidant, certain characteristics must be observed. First, it must be nontoxic and have good interaction with other antioxidants. Still, it must have a high scavenging capacity, even at low concentrations in the medium. Its power of action in different media, based on solubility, must be satisfactory (14,28). There should not be modifications of the original organoleptic characteristics of the product, which must be compatible with the formulation and general processes of the product (29). Finally, it must have a long half-life and acceptable bioavailability, with the ability to

cross biological membranes and reach their place of action (27). These characteristics make it difficult to obtain an ideal antioxidant for use in the industry, and although there are various synthetic antioxidant compounds, such as butyl hydroxyanisole (BHA), butylhydroxytoluene (BHT), t-butyl hydroquinone (TBHQ), and propyl gallate (PG), their possible toxic and carcinogenic side effects guide research to natural targets to reduce the risks (1,9).

Furthermore, numerous studies linking the consumption of fruits and other foods with high levels of antioxidants with the low risk of disease have raised population interest in these compounds (1). Consumers seek not only nutritional quality but also foods that promote quality in health (30).Antioxidant substances of natural origin can greatly impact diseases such as diabetes mellitus, obesity,and high blood pressure (31).

In this context, interest in propolis has grown recently. The huge number of studies involving this natural product aims to understand its various biological activities and the properties of each chemical constituent. These studies help build a standardization of reference for producers who use this raw material, increasing its economic value and becoming a source of new drugs (32). Then, this work investigates the antioxidant activity of *Apis mellifera* bee propolis extracts through a literature review to contribute to the field of health and natural products.

METHODS

The design that guided this literature review was based on the following steps: determining the theme, objective, and information to be obtained from each material, the establishment of inclusion and exclusion criteria, choice of databases, sample selection, analysis of materials found, discussion, and presentation of results in the form of a review.

Eligibility Criteria

Inclusion Criteria

Experimental works (articles, theses, and dissertations) that present tests that evaluate the antioxidant activity of *Apis mellifera* propolis, *in vivo* and *in vitro*, were eligible.

Exclusion Criteria

The following works were excluded: (1) Studies outside the specified period (2011 to 2021); (2) Studies with inappropriate format (reviews, books, chapters, conference abstracts and posters, letters, and opinion articles); (3) Studies written in languages other than Portuguese, English, and Spanish; (4) Studies that have not tested the antioxidant activity; (5) Studies that did not use propolis as study material; (6) Studies that did not specify the propolis-producing bee species; (7) Studies that used other bee species, except *Apis mellifera*; (8) Studies that did not specify the extract preparation process or the propolis collection location; (9) Studies that did not use the crude propolis extract, but partitioned, isolated substances, or used co-products and residues; (10) Studies that evaluated the antioxidant activity of propolis in association with other substances.

Information Sources and Search Strategy

The databases chosen for the search were SciELO (Scientific Electronic Library Online), PubMed, BVS (Virtual Health Library – *Biblioteca Virtual em Saúde*), Scopus, Cochrane Library, Embase, Science Direct, CRD (Center for Reviews and Dissemination), and CAPES Theses and Dissertations Catalog, to ensure adequate coverage on the subject.

Two searches were performed in each aforementioned database: the first, with the term in English and the second in Portuguese (Suplemmentary Material – Table S1); although articles in Spanish that eventually appeared in the searches were accepted, there was no direct search for terms in Spanish. Within each database, the exclusion criterion by date has already been performed.

All terms were consulted on health descriptors through the DeCS website (Descriptors in Health Sciences), in addition to the MESH search. The terms "*Apis mellifera*" and “propolis” were duly identified. However, "antioxidant activity" was not found in DeCS/MESH.

Study Selection

This literature review comprised four steps. In the first, a search was performed according to the appropriate terms in each database. Later, in the second stage, such references were added to the EndNote Web citation management program, where duplicate removal was performed. During the third stage, works that did not meet the above criteria were removed by reading the title and abstract. Finally,

the remaining references were analyzed again in the fourth stage, but this time through a full reading of the text.

Information was taken from the materials that make up this review: type of material (article, theses, and dissertation), year of publication, language, type of propolis used, time and place of propolis collection, species and nomenclature of the bee, the type of extract, a type of methodology used to assess the antioxidant activity, and plant source of propolis.

RESULTS

Initially, 1765 publications were identified in the first phase from January 1st, 2011, to April 29th, 2021. After insertion in the manager EndNote Web, 242 duplicates were deleted. Of the 1523 remaining materials, 1239 were eliminated after analyzing the title and abstracts, as it was perceived that they did not meet the pre-established criteria. The remaining 284 articles formed the initial collection, whose later reading would be complete. A full-text analysis of each material eliminated another 111 articles. Finally, this review was based on data obtained from 173 scientific articles, theses, and dissertations, which form the analytical field, as shown in Figure 01.

Study Characteristics

The more specific characteristics, as well as qualitative information (reference, bee race, place and time of collection of propolis and its type, sample extraction process, and possible botanical source) of each work chosen for this literature review, can be found is Supplementary Material (Table S2).

Regarding the year of publication, there is an increase in interest in the topic, with a peak in the bienniums of 2016/17 and 2019/20. 2021 could not be analyzed clearly, because this survey was conducted only with works published until the end of April. However, it is noted that, even if the search is conducted in the first half of 2021, this year still had more results compared to 2012 and 2013 (Figure 02).

One hundred forty-four scientific articles (83.24%) and 29 theses/dissertations (16.76%) were identified, mainly published in English (80.92%), but there were also works in Portuguese (17.34%) and Spanish (1.73%).

DISCUSSION

Antioxidant Activity

The antioxidant activity can be defined as the redox capacity to eliminate oxidizing compounds. Numerous studies prove the antioxidant action of propolis samples – according to Castro*et al*. (2014), it has the highest activity among all hive products (34). This power is mainly due to its chemical composition rich in bioactive compounds, such as flavonoids and other phenolic compounds(1,7).

The quality control of propolis is done based on the extraction yield, number of phenolic compounds, and analysis of the antioxidant activity of this material (12). This essential activity is one of the main factors for propolis to have many other uses, such as neuro and hepatoprotection. One of the most viable applications of this antioxidant power is in the treatment of skin wounds, remembering that the intensified production of free radicals makes it difficult for these inflammatory processes to heal (7). Still, this ability of propolis can be used for treating other illnesses, such as glaucoma, ocular neuropathies resulting from diabetes complications, and cerebral ischemia, since all these cases are aggravated by oxidative stress (35).

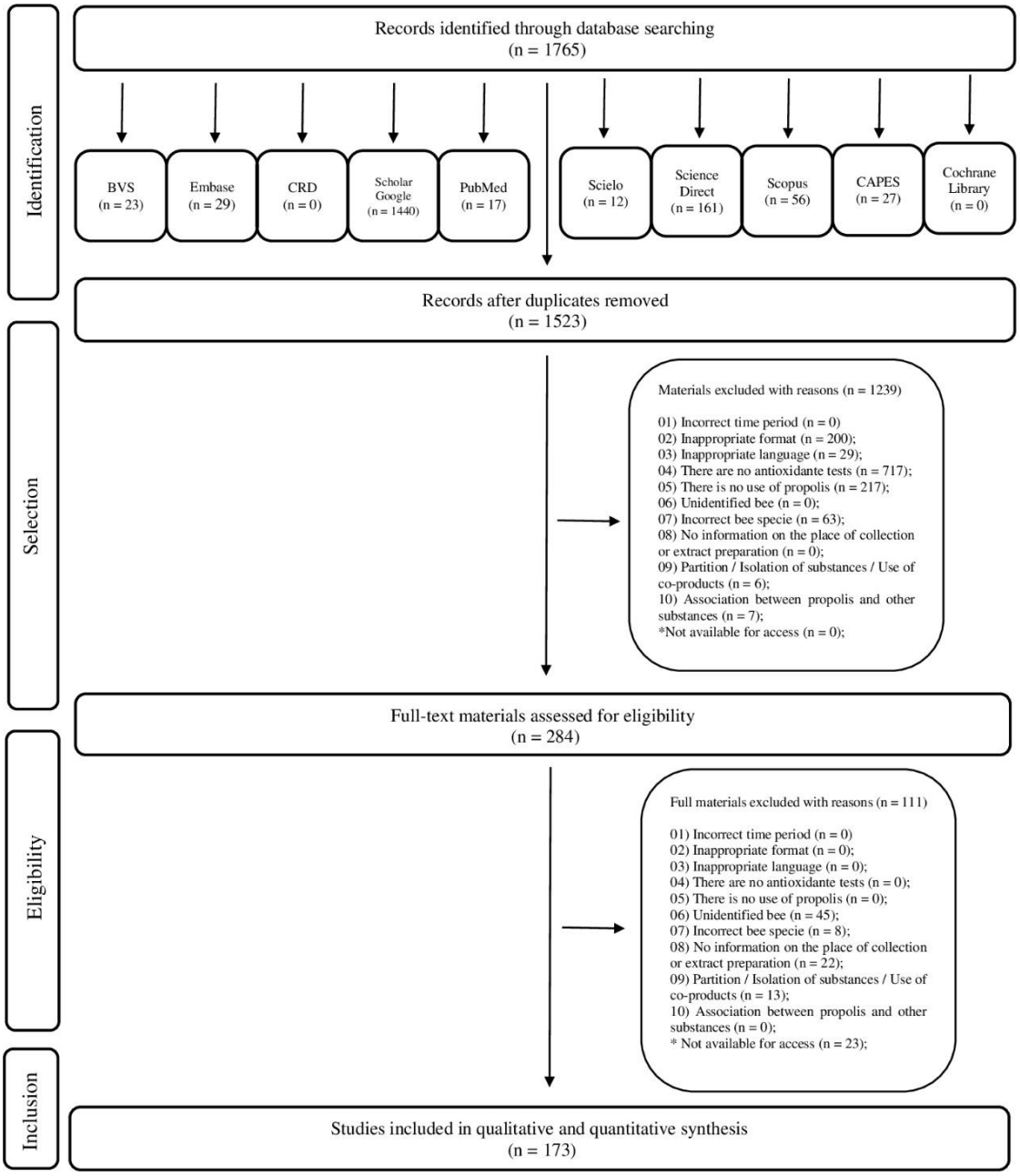

Figure 01 | Flowchart of materials obtained, excluded, duplicated, and used in this literature review. Adapted from PRISMA (33).

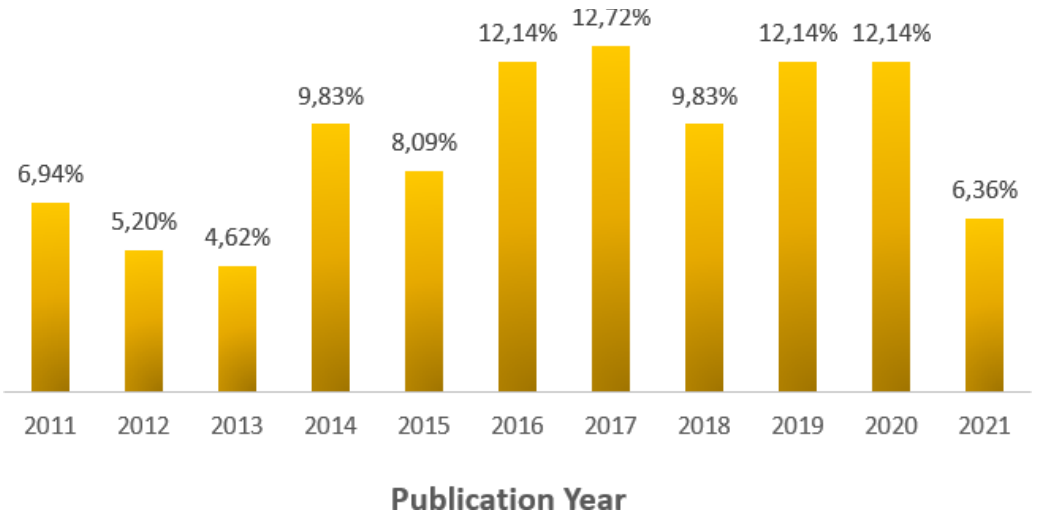

Figure 02 | The year of publication of the materials that make up the collection of this bibliographic review.

There are several methods for evaluating antioxidant capacity, both *in vitro* and *in vivo*, since the samples to be analyzed also differ greatly from each other. Those *in vitro* tests can be performed using instrumental, colorimetric, biological, and electrochemical tests (29). Generally, studies bring more than one method for evaluating the same sample, giving greater credibility to the data. When looking at the results obtained using different methodologies, there are differences. This fact is due to the reaction mechanism and the conditions under which radicals are generated (36). Each methodology has advantages, disadvantages, and limitations, with different reaction mechanisms, complexes, solubility, and compounds. To choose the best method of study, the sample matrix, a form of preparation, and antioxidant solubility should be considered (12,26,35).

This literature review analyzed antioxidant activity by *in vitro* (93.75%), *in vivo* (5.11%), and 1.14% mixed both forms. 29.71% of the materials used only one antioxidant test to evaluate the propolis samples, generally the DPPH; the vast majority (70.29%) opted for more than one analysis methodology. The works that included *in vitro* methodologies analyzed enzymatic activity, protein oxidation levels, lipid peroxidation by the TBARS method (Thiobarbituric Acid Reactive Substances), the production of ROS (Reactive Oxygen Species), cell survival, in addition to tissue histological analysis.

Some studies have obtained remarkable results for the antioxidant activity of propolis samples, with similar or even better response than the used reference compounds, widely recognized as good antioxidants, such as quercetin and BHT.

More detailed results of the works that comprise the collection of this review will be presented below, focusing on the values obtained for the antioxidant activity. The tables were organized to show the material reference and the values of the results of the respective tests. Those that appear together with the term “about” were values taken from graphs whose specific numbers were not reported in the materials.

DPPH• (2,2-Diphenyl-1-Picrylhydrazyl) Radical Scavenging Test

Initially suggested in 1950 by Blois, DPPH is considered an indirect and ancient method for evaluating the antioxidant activity, extremely used, practical and simple, stable in the absence of light, and very sensitive (29,37). It is considered a good method to evaluate bee products (9).

One of the ways to demonstrate the result obtained by this method is through the so-called IC_{50} (Half-inhibitory Concentration), which corresponds to the amount of sample required to reduce 50% of the initial DPPH. The final evaluation can be interpreted considering that the greater the consumption of DPPH, the greater the antioxidant activity of the sample. Another way to express the results is by the percentage of inhibition (9).

Some authors expressed the results in the form of an index of antioxidant activity (IAA), a scale that classifies the action of compounds as weak (IAA<0.50), moderate (IAA between 0.50 and 1.00), strong (IAA between 1.00 and 2.00) and very strong (IAA>2.00) (38). Moreover, this methodology can be performed on a silica plate more simply. In this case, the sample is applied to the plate, followed by spraying a solution of DPPH in methanol. If there is a change in color from purple to yellow, the result is considered positive, demonstrating the antioxidant potential of the analyzed sample (39).

Table 1 shows the values obtained from the collection of chosen works. Generally, the propolis samples showed remarkable antioxidant power; their IC_{50} values approached or were even better than the chosen antioxidant standards, such as ascorbic acid, which already has recognized potency in these tests (13,34,40).

Table 1 | Results of the DPPH radical scavenging test of *Apis mellifera* propolis extracts, obtained from the materials chosen for this literature review.

Author	Percentage of inhibition (%)	Other results
Aguiar, 2015 (10)	97.20% (0.10 mg/mL) and 99.80% (1.00 mg/mL)	IC_{50}: 24.20 µg/mL
Al Naggar *et al*., 2016 (42)	64.64 – 88.15% (25.00 µg/mL); 81.93 – 92.10% (50.00 µg/mL); 91.19 – 93.72% (100.00 µg/mL)	-
Alves, 2018 (5)	EEP: 88.37% Microcapsules: 73.41 – 86.93%	-
Andrade *et al*., 2017 (43)	-	4431.00 – 4663.80 µg Trolox/g
Andrade *et al*., 2018 (36)	82.00 – 89.00%	22843.03 - 24685.82 µmol Trolox/g
Aranguena Salazar, 2019 (44)	-	642.99 – 828.55 µg Trolox/g
Araújo *et al*., 2020 (45)	-	IC_{50}: 100.18 µg/mL
Arruda, 2019 (46)	78.50 – 81.5% (80.00 µg/mL)	IC_{50}: 3.10 – 51.03 µg/mL
Arslan *et al*., 2021 (47)	94.60% and 94.90%	-
Augusto-Obara *et al*., 2019 (48)	-	682.80 – 781.20 µmol Trolox/g
Bakkaloglu, Arici, and Karasu, 2021 (49)	-	0.48 – 835.34 mg Trolox/g
Béji-Srairi *et al*., 2020 (50)	-	IC_{50}: 20.10 – 43.00 µg/mL
Bhargava *et al*., 2014 (51)	-	59.30 and 81.40 g/mL (5.00 and 10.00%)
Bhuyan *et al*., 2021 (52)	4.85 – 75.02% (6.25 to 100.00 µg/mL)	IC_{50}: 52.63 - >100.00 µg/mL
Bonamigo *et al*., 2017 (4)	94.60% (300.00 µg/mL)	IC_{50}: 49.80 µg/mL
Boufadi *et al*., 2014 (53)	-	IC_{50}: 19.40 - >50.00 µg/mL
Cabral *et al*., 2012 (54)	Type 06 propolis: 21.70% Type 12 propolis: 53.00%	-

Calegari, 2018 (26)	-	136.00 – 267.00 µmol Trolox/g
Cao *et al*., 2017 (7)	-	IC_{50}: 47.71 µg/mL
Castro *et al*., 2014 (34)	-	About IC_{50}: 5.00 – 12.00 µg/mL
Cavalaro, Fabricio, and Vieira, 2020 (55)	-	18652.90 µmol Trolox/g
Cécere *et al*., 2021 (56)	-	IC_{50}: 158.15 µg/mL
Ceylan, and Halime, 2020 (57)	-	IC_{50}: 3.94 – 26.33 mg/mL
Coelho, 2013 (58)	-	IC_{50}: 20.00 – 110.00 µg/mL
Coelho *et al*., 2017 (20)	-	IC_{50}: 10.00 – 110.00 µg/mL
Correa *et al*., 2016 (59)	-	IC_{50}: 7.81 and 37.01 µg/mL (some samples were not active)
Cottica *et al*., 2011 (60)	-	IC_{50}: 47.00 – 160.00 µg/mL
Cruz, 2011 (61)	-	50.46 mg of equivalents of gallic acid/g
Da Cruz Almeida *et al*., 2017 (62)	EEP: 16.52 – 98.06% (2.50 – 50.00 µg/mL) Microcapsules: 5.89 – 87.86% (2.50 – 50.00 µg/mL)	IC_{50} EEP: 6.95 and 4.78 µg/mL IC_{50} Microcapsules: 8.89 – 23.36 µg/mL
Da Graça Miguel *et al*., 2014 (63)	-	IC_{50}: 8.00 – 1813.00 mg/mL
Dărăban *et al*., 2019 (64)	-	IC_{50}: 1.30 – 3.70 µL
Da Silva *et al*., 2013 (65)	24.00 – 52.00% (500 and 600 ppm); 62.00 – 81.00% (2000 ppm); 84.00 – 89.00% (5000 ppm)	-
Da Silva *et al*., 2018 (66)	-	11.68 – 275.20 µmol Trolox/g
De Almeida, 2017 (67)	EEP: 74.01 – 96.72% Microcapsules: 53.88 – 92.56%	-
De Francisco *et al*., 2018 (30)	-	IC_{50}: 913.18 µg/mL
De Lima *et al*., 2019a (25)	-	IC_{50}: 25.00 µg/mL
De Lima *et al*., 2019b (68)	-	IC_{50}: 9.35 µg/mL
De Mendonça, 2014 (69)	10.91% (50.00 µg/mL); 13.33% (100.00 µg/mL); 22.10% (150.00 µg/mL); 31.82% (200.00 µg/mL); 40.46% (250.00 µg/mL)	IC_{50}: 372.44 µg/mL

De Oliveira, and Andolfatto, 2014 (70)	34.65 – 57.68%	3.16 – 8.75 µmol Trolox/g
De Souza *et al*., 2018 (71)	-	IC_{50}: 3.74 µg/mL
Devequi-Nunes *et al*., 2018 (72)	-	IC_{50} EEP: 89.90 – 159.74 µg/mL IC_{50} Supercritical: 141.81 – 371.12 µg/mL
Ding *et al*., 2021 (73)	About 80.00 – 95.00% (5.00 mg/mL)	-
Do Nascimento *et al*., 2016 (74)	EEP: 98.00% Nanoparticles: 76.22 – 81.40%	-
Do Nascimento *et al*., 2019 (75)	EEP: 91.07% Microcapsules: 90.60%	-
Duca *et al*., 2019 (76)	84.71 – 92.57 % (10.00 mg/mL); 79.57 – 92.50% (5.00 mg/mL); 72.63 – 92.63% (3.00 mg/mL); 67.58 – 90.66% (1.50 mg/mL); 13.58 – 82.52% (0.50 mg/mL); 7.89 – 78.16% (0.30 mg/mL);	IC_{50}: 70.00 – 932.00 µg/mL
El Meniiy *et al*., 2021 (77)	-	IC_{50}: 21.00 – 1308.00 µg/mL
El Sohaimi, and Masry, 2014 (78)	8.98 – 99.20% (5.00 - 200.00 µg/mL)	IC_{50}: 73.49 and 81.67 µg/mL
Ezzat *et al*., 2019 (79)	12.93 – 91.42% (10.00 – 1280.00 µg/mL)	IC_{50}: 49.70 µg/mL
Falcão, 2013[1] (13)	-	IC_{50}: 8.00 – 93.00 µg/mL
Fangio *et al*., 2019 (80)	-	566.00 – 1477.00 µmol Trolox/g
Farias Azevedo *et al*., 2018 (81)	EEP: 79.10 – 95.70% (2.50 – 80.00 µg/mL); Nanoparticles: 58.90 – 100.00% (2.50 – 80.00 µg/mL)	-
Ferreira, 2015 (2)	4.46 – 17.95% (15.00 µg/mL); 6.85 – 29.66% (30.00 µg/mL); 8.23 – 40.77% (45.00 µg/mL); 9.66 – 52.18% (60.00 µg/mL)	-
Ferreira, 2017 (6)	-	About 120.00 – 310.00 µmol of equivalents of ascorbic acid/g
Ferreira, 2019 (82)	-	IC_{50}: 56.20 and 72.90 µg/mL
Ferreira *et al*., 2017 (83)	-	IC_{50}: 56.20 and 72.90 µg/mL
Fianco, 2014 (8)	-	IC_{50} Green propolis: 0.05 – 0.09 g of extract/g of DPPH IC_{50} Red propolis: 0.08 – 0.10 g of extract/g of DPPH

Galeotti *et al*., 2017 (84)	-	About 28.00 – 57.00 µg Trolox
Gonçalves, Santos, and Srebernich, 2011 (9)	60.93% (0.10%)	505.14 mg/L or 38.13 g of sample/g of DPPH
Guo *et al*., 2011 (85)	-	IAA: 0.28 – 3.29
Hames-Kocabas *et al*., 2013 (86)	-	IC_{50}: 4.95 – 37.09 µg/mL
Ikeda, 2020 (87)	-	EEP: 0.15 µmol Trolox/mg Essential oil: No activity
Irigoiti, Yamul, and Navarro, 2021 (88)	-	About 0.75 – 2.00 µg Trolox/g
Jansen, 2015 (40)	4.55 – 38.32% (25.00 µg/mL); 13.33 – 90.66% (125.00 µg/mL); 36.06 – 91.35% (250.00 µg/mL)	IC_{50}: 50.35 – 108.69 µg/mL (some samples did not reach IC_{50})
Jiang *et al*., 2020 (89)	-	IC_{50}: 71.19 – 432.08 µg/mL
Jug; Končić; Kosalec, 2014 (90)	-	IC_{50}: 29.00 -114.40 µg/mL
Kasote *et al*., 2017 (12)	-	0.29 – 0.38 mmol Trolox/mg
Kumar *et al*., 2011 (91)	-	IC_{50}: 75.00 µg/mL
Kumazawa *et al*., 2013 (21)	23.00 – 85.70%	-
Kumul *et al*., 2020 (92)	4.13 – 67.32%	-
Kunrath *et al*., 2017 (93)	-	IC_{50} EEP: 89.94 g of propolis/g of DPPH IC_{50} Dry extract: 5.86 g of propolis/g of DPPH
Kurek-Górecka *et al*., 2012 (94)	-	IC_{50}: 256.86 and 268.60 mg
Labyad *et al*., 2016 (39)	Plate methodology: positive	-
Lacerda, 2012 (35)	-	4.50 – 148.10 µmol Trolox/g
Luis-Villaroya *et al*., 2015 (95)	-	IC_{50}: 55.00 µg /mL
Machado *et al*., 2016 (96)	-	IC_{50} EEP: 31.80 – 273.46 IC_{50} Supercritical: 85.34 – 373.53
Marcussi, and Gutierrez-Gonçalves, 2013 (97)	-	IC_{50}: 14.83 – 56.29 µg/mL
Masek *et al*., 2019 (98)	About 20.00 – 85.00% (0.50 – 3.00 mg/mL)	-

Mello, and Hubinger, 2012 (99)	About EEP: 52.00 – 88.00% WEP: 25.00 – 45.00%	-
Mendez-Pfeiffer *et al*, 2020 (100)	-	IC_{50}: 58.80 – 98.70 µg/mL
Miguel *et al*., 2014 (101)	Winter: 35.76 – 92.28% Spring: 38.16 – 91.55%	-
Mohdaly *et al*., 2015 (102)	28.50% (5.00 µg/mL) and 85.00% (25.00 µg/mL)	-
Molnár *et al*., 2017 (103)	-	64.10 – 214.20 mg of equivalents of ascorbic acid/g
Moncayo Luján *et al*., 2018 (104)	11.40 – 12.39%	-
Naik, and Vaidya, 2011 (105)	17.10 – 81.09 (0.10 – 2.00 mg/mL)	IC_{50}: 320.00 µg/mL
Nina *et al*., 2015 (106)	-	IC_{50}: 10.29 – 91.84 µg/mL (some samples were inactive)
Nina *et al*., 2016 (107)	-	IC_{50}: 4.54 – 58.71 µg/mL
Nori *et al*., 2011 (108)	15.97 and 21.65% (600.00 ppm); 53.37 and 56.40% (1500.00 ppm); 84.10 and 84.94% (3000.00 ppm)	-
Oldoni *et al*., 2015 (109)	-	31.60 – 87.50 µg Trolox/g
Ozdal *et al*., 2018 (110)	-	391.73 mg Trolox/g
Ozdal *et al*., 2019 (111)	-	13.71– 63.33 mg Trolox/g
Pazin *et al*., 2017 (112)	-	IC_{50} (optical absorbance): 32.4 µg/mL IC_{50} (electronic spin resonance): 9.50 µg/mL
Peixoto *et al*., 2021 (18)	-	IC_{50}: 11.80 – 13.70 µg/mL
Permana *et al*., 2020 (113)	-	IC_{50}: 43.29 – 863.44 µg/mL
Prasniewski, 2015 (114)	-	11.68 – 175.77 µmol Trolox/g
Quintino *et al*., 2020 (115)	-	IC_{50}: 23.48 µg/mL
Ramnath, and Venkataramegowda, 2016 (116)	-	IC_{50}: 333.48 – 600.88 µg/mL
Righi *et al*., 2011 (117)	30.62% (8.00 µg/mg); 32.75% (12.50 µg/mg); 39.12% (25.00 µg/mg);	-

Ristivojević *et al*., 2018 (118)	26.49 – 65.64%	-
Ristivojević *et al*., 2020 (119)	40.51 and 53.21%	-
Rivera-Yañez *et al*., 2018 (120)	-	IC_{50}: 15.75 µg/mL
Rocha *et al*., 2013 (121)	-	IC_{50}: 33.36 and 56.71 µg/mL
Rodríguez *et al*., 2012 (122)	-	65.10 – 190.40 µmol Trolox/g
Rodríguez Pérez *et al*., 2020 (123)	-	IC_{50}: 26.00 – 950.40 µg/mL
Salgueiro, 2016 (37)	-	IC_{50} EEP: 30.65 – 56.71 µg/mL IC_{50} MEP: 6.93 – 23.69 µg/mL IC_{50} Soxhlet: 15.10 – 72.44 µg/mL
Salgueiro, and Castro, 2016 (124)	-	IC_{50}: 30.65 – 56.71 µg/mL
Sánchez *et al*., 2020 (125)	11.50 – 72.30%	-
Sanpa *et al*., 2017 (41)	-	IC_{50}: 19.90 – 67.20 µg/mL
Saral *et al*., 2019 (126)	-	IC_{50}: 20.00 – 130.00 µg/mL
Schmidt *et al*., 2014a (127)	-	IC_{50}: 17.13 – 83.60 µg/mL
Schmidt *et al*., 2014b (128)	-	IC_{50}: 29.00 – 87.83 mg/L
Shahbaz *et al*., 2021 (129)	WEP: 44.73% EEP: 61.26 – 73.18 % MEP: 59.01 – 70.06%	-
Shehata *et al*., 2020 (130)	About 28.00 – 90.00% (1.00 mg/mL)	-
Shubharani, Mahesh, and Yogananda Murti, 2019 (131)	-	IC_{50}: 78.90 – 358.20 µg/mL
Silva *et al*., 2018 (132)	92.40% (250.00 µg/mL); 22.10% (5.00 µg/mL); DPPH in plate: positive	IC_{50}: 3.97 µg/mL
Sime *et al*., 2015 (133)	48.60 – 87.80%	247.00 – 455.00 mg of equivalents of ascorbic acid/g
Sousa *et al*., 2019 (134)	-	IC_{50}: 11.46 – 77.30 µg/mL

Sulaiman *et al*., 2011 (135)	20.00 – 63.30% (1.00 µg/mL); 26.60 – 76.60% (10.00 µg/mL); 40.00 – 83.30% (100.00 µg/mL)	-
Sun *et al*., 2015 (136)	-	IC_{50} WEP: 13798.00 µg/mL IC_{50} EEP: 633.00 – 7129.00 µg/mL
Svečnjak *et al*., 2020 (137)	-	2.60 – 81.60 mg of equivalents of gallic acid/g
Tiveron, 2015 (27)	-	4.47 – 148.06 µmol Trolox/g
Tiveron *et al*., 2016 (138)	-	0.01 – 0.38 mg Trolox/g
Toreti, 2011 (139)	20.59 – 72.84%	1628.00 – 126164.06 µmol Trolox/g
Touzani *et al*., 2018 (140)	-	IC_{50}: 19.00 – 1190.00 µg/mL
Vargas-Sánchez *et al*., 2019 (141)	28.70 – 69.10% (12.50 – 500.00 µg/mL)	-
Vargas-Sánchez; Torrescano-Urrutia; Sánchez Escalante, 2020 (142)	83.20 – 85.10% (250,00 µg/mL)	-
Venegas *et al*., 2016 (143)		IC_{50}: 14.28 – 43.08 µg/mL
Wali *et al*., 2016 (144)	-	IC_{50}: 76.15 – 102.17 µg/mL
Wang *et al*., 2016 (145)	-	IC_{50}: 43.40 – 269.00 µg/mL
Wiwekowati *et al*., 2017 (146)	-	IC_{50}: 35.60 µg/mL
Xavier *et al*., 2017 (147)	14.80 and 44.70%	IC_{50}: 33.10 and 78.50 µg/mL
Yuan *et al*., 2020 (148)	-	IC_{50}: 34.61 and 47.28 µg/mL
Yurteri, 2015 (149)	96.00% (10.00 mg/mL)	IC_{50}: 4101.00 µg/mL
Zeitoun *et al*., 2019 (150)	-	IC_{50}: 122.80 µg/mL
Zhang *et al*., 2015 (151)	-	IC_{50}: 32.35 µg/mL
Zhang *et al*., 2016 (152)	-	IC_{50}: 19.55 – 43.85 µg/mL
Žižić *et al*., 2013 (153)	-	IC_{50}: 55.45 – 118.46 µg/mL

EEP: Ethanol Extract of Propolis; WEP: Water Extract of Propolis; MEP: Methanol Extract of Propolis

ORAC (Oxigen Radical Absorbance Capacity)

The ORAC Index (Oxygen Radical Absorbance Capacity), or evaluation of the antioxidant activity by oxygen radical absorption, is performed, *in vitro*, to measure the capacity antioxidant of a compound or its power to scavenge the peroxide radical through the transfer of a hydrogen atom (154).

Among the chosen works, the results of Castro *et al*. (2014) demonstrate variations between the components and the antioxidant capacity of the samples, although all have been collected in the same

city. These results reinforce the idea that propolis has extremely varied activities and chemical composition, with high dependence on external factors and the genetics of the hive (34).

The other ORAC test results arising from the materials of this review are presented in Table 02.

Table 2 | Results of the ORAC test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Results
Andrade *et al*., 2017 (43)	5343.84 – 6734.87 µmol Trolox/g
Andrade *et al*., 2018 (36)	4332.73 – 6261.96 µmol Trolox/g
Castro *et al*., 2014 (34)	8.90 – 33.10 µmol of equivalents of caffein acid/mg
Cavalaro, 2017[1](29)	2210.20 – 3524.00 µmol Trolox/g
Cavalaro, 2017[2](29)	21.30 – 13244.50 µmol Trolox/g
Correa *et al*., 2016 (59)	1352.16 – 5769.72 µmol Trolox/g (some samples were inactive)
Da Graça: Miguel *et al*., 2014 (63)	1106.42 – 2012.15 µmol Trolox/g
De Morais *et al*., 2021 (154)	4339.61 µmol Trolox/g
Lacerda, 2012 (35)	0.18 - 1.25 µmol Trolox/g
Pandolfo, 2014 (1)	150.00 – 4520.00 µmol Trolox/g
Rodríguez *et al*., 2012 (122)	475.00 – 2211.90 µmol Trolox/g
Silva *et al*., 2011 (155)	1800.00 – 9000.00 µmol Trolox/g
Sun *et al*., 2015 (136)	WEP: 138.50 µmol Trolox/g EEP: 918.00 – 27595.40 µmol Trolox/g
Tiveron, 2015 (27)	500.00 – 1950.00 µmol Trolox/g
Tiveron *et al*., 2016 (138)	500.00 – 1950.00 µmol Trolox/g
Vargas Tapia, 2018 (156)	About 50.00 – 267.48 µmol Trolox/mL
Zhang *et al*., 2015 (151)	9250.00 µmol Trolox/g
Zhang *et al*., 2016 (152)	5600.00 – 9250.00 µmol Trolox/g

EEP: Ethanol Extract of Propolis; WEP: Water Extract of Propolis.

FRAP (Ferric Reducing Antioxidant Power)

This colorimetric test, called the antioxidant test for determining the power of iron ion reduction, has the advantage of being able to use complex samples, biological fluids, and pure substances in aqueous solutions, in addition to being relatively simple, with easy standardization (34,85).

Phenolic compounds are the class of compounds with the greatest biological activity of propolis, especially in terms of antioxidant activity. As far as the FRAP methodology is concerned, the same applies. These molecules have great power to reduce iron ions, due to their ability to donate electrons. The samples that have the highest number of these compounds are those that obtain the best results in this methodology (40).

The results of the studies are shown in Table 03. Again, in most cases, the propolis samples performed similarly or better than the commercial pure antioxidants.

Table 3 | Results of the FRAP test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Results
Abubaker, and Fageer, 2017 (157)	3790.00 and 36530.00 µmol of equivalents of vitamin C/mL
Alves, 2018 (5)	EEP: 815.75 µmol Trolox/g Microcapsules: 34.14 – 139.31 µmol Trolox/g
Andrade *et al*., 2017 (43)	471.51 – 633.18 µmol Trolox/g
Andrade *et al*., 2018 (36)	144.87 – 396.09 µmol Trolox/g
Arslan *et al*., 2021 (47)	3813.20 and 4017.70 µmol Iron/g
Béji-Srairi *et al*., 2020 (50)	IC_{50}: 375.00 – 780.00 µg/mL
Bhargava *et al*., 2014 (51)	1.97 and 3.75 mg/mL (5.00 and 10.00%)
Calegari, 2018 (26)	1571.00 – 1830.00 µmol Iron/g
Calegari *et al.,* 2017 (3)	2013: 89.70 – 286.70 µmol Iron/g 2015: 638.1 – 1041.0 µmol Iron/g
Castro *et al*., 2014 (34)	1700.00 – 3200.00 µmol of equivalents of cafeic acid/g
Cavalaro, Fabricio, and Vieira, 2020 (55)	36231.00 µmol Iron/g
Cottica *et al*., 2011 (60)	528.00 - 1365.00 µmol Iron/g
Dărăban *et al*., 2019 (64)	51.96 – 93.79 µmol Trolox/mL
Da Silva *et al*., 2018 (66)	66.74 – 1164.00 µmol Iron/g
De Francisco *et al*., 2018 (30)	1536.40 µmol Iron/g
De Morais *et al*., 2021 (154)	1472.86 µmol Iron/g
De Oliveira, and Andolfatto, 2014 (70)	60.67 µmol Iron/g
Ding *et al*., 2021 (73)	126.00 – 290.34 µmol Iron/g
Ertürk *et al*., 2016 (158)	361956.00 mmol FRAP
Ferreira, 2017 (6)	About 200.00 – 1200.00 µmol Iron/g
Gokduman, 2019 (159)	247.64 mg Trolox/g
Ikeda, 2020 (87)	EEP: 1386.14 µmol Iron/mg Essential oil: 183.98 µmol Iron/mg
Jansen, 2015 (40)	750.00 – 4670.00 µmol Iron/g
Kasote *et al.,* 2017 (12)	130000.00 – 7340000.00 µmol Trolox/g
Kiziltas, and Erkan, 2020 (160)	1.54– 5.98 µmol Trolox/g
Kunrath *et al.,* 2017 (93)	EEP: 50.98 µmol Iron/g Dry extract: 543.40 µmol Iron/g
Masek *et al*., 2019 (98)	About 0.75 – 2.50 (absorbance difference)
Mello, and Hubinger, 2012 (99)	EEP: 219.04 – 2078.57 µmol Iron WEP: 180.95 – 1308.09 µmol Iron

Nina *et al*., 2015 (106)	667.43 – 1754.03 µmol Trolox/g (some samples were inactive)
Nina *et al*., 2016 (107)	246260.00 – 905950.00 µmol Trolox/kg (some samples were inactive)
Oldoni *et al*., 2015 (109)	259.30 µmol Iron/g
Ozdal *et al*., 2018 (110)	156.59 mg Trolox/g
Prasniewski, 2015 (114)	66.74 – 837.17 µmol Iron/g
Rodríguez *et al*., 2012 (122)	112.10 – 321.30 µmol of equivalents of ascorbic acid/g
Salgueiro, 2016 (37)	EEP: 2607.90 – 6088.50 mmol Iron/g MEP: 4137.90 – 14147.20 mmol Iron/g Soxhlet: 2606.20 – 4907.90 mmol Iron/g
Salgueiro, and Castro, 2016 (124)	2607.90 – 6088.50 mmol Iron/g
Sánchez *et al*., 2020 (125)	1.00 – 7.20 mg Iron/g
Saral *et al*., 2019 (126)	166.91- 1600.25 µmol de Iron/g
Shubharani, Mahesh, and Yogananda Murti, 2019 (131)	IC_{50}: 2.86 – 5.71 µg/mL
Sousa *et al*., 2019 (134)	2075.50 – 3472.00 mmol Iron/g
Sun *et al*., 2015 (136)	WEP: 20.00 µmol Trolox/mg EEP: 16.00 – 233.00 µmol Trolox/mg
Svečnjak *et al*., 2020 (137)	100.00 – 800.00 µmol Iron/g
Touzani *et al*., 2018 (140)	IC_{50}: 39.00 – 1080.00 µg/mL
Vargas Tapia, 2018 (156)	About 18.00 - 49.30 µmol Iron/mL
Xavier *et al*., 2017 (147)	42.00 and 157.60 mg Trolox/g

EEP: Ethanol Extract of Propolis; WEP: Water Extract of Propolis; MEP: Methanol Extract of Propolis.

Reducing Power (RP)

Similar to the FRAP method, the test called “reducing power” (RP) also explores the ability to reduce iron ions through electron donation (13). The results of this methodology found in the works of this literature review are presented below, in Table 04.

Table 4 | Results of the RP test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Results
Cao *et al.,* 2017 (7)	1.73 mmol Trolox/g
Coelho, 2013 (58)	0.07 – 0.99 g of equivalents of caffeic acid: galangin: pinocembrin (1:1:1)/g
Coelho *et al.,* 2017 (20)	0.09 – 0.68 g of equivalents caffeic acid: galangin: pinocembrin (1:1:1)/g
Da Graça Miguel *et al*., 2014 (63)	About; there was an increase in absorbance (0.25 – 2.60) at the concentration of 1.00 mg/mL

Ding *et al*., 2021 (73)	About 0.12 – 0.35 (5.00 mg/mL)
El Meniiy *et al*., 2021 (77)	IC_{50}: 42.00 – 1512.00 µg/mL
Falcão, 2013[1](13)	110.00 – 757.00 mg of equivalents of caffeic acid: galangin: pinocembrin (1:1:1)/g
Guo *et al*., 2011 (85)	1.20 – 3.47 (K value: increase in absorbance)
Mouhoubi-Tafinine, Ouchemoukh, and Tamendjari, 2016 (24)	About 1.00 – 25.00 mg of equivalents of galic acid/g
Rocha *et al*., 2013 (121)	IC_{50}: 270.00 and 282.00 µg/mL
Shehata *et al*., 2020 (130)	About 0.20 – 0.90 (1.00 mg/mL)
Vargas-Sánchez *et al*., 2019 (141)	0.07 – 0.56% (12.50 – 500.00 µg/mL)
Vargas-Sánchez; Torrescano-Urrutia; Sánchez Escalante, 2020 (142)	>50.00% of inhibition (125.00 and 250.00 µg/mL)
Wali *et al*., 2016 (144)	Approximate absorbance from 1.00 to 1.80 (concentration of 150.00 µg/mL)
Zhang *et al*., 2015 (151)	2.08 mmol Trolox/g
Zhang *et al*., 2016 (152)	1.53 – 2.70 mmol Trolox/g

CUPRAC (Cupric Ion Reducing Antioxidant Capacity)

The CUPRAC method is based on measuring the reducing capacities of substances using the reduction of copper ions (23). It is considered cheap, fast (30 minutes), simple and versatile, able to analyze several compounds, such as polyphenols, flavonoids, vitamins, and synthetic antioxidants. Results are generally expressed in Trolox equivalents (161).

Compared to other methods, it is observed that CUPRAC presents higher antioxidant capacity values, mainly due to the difference in solubility between the solvents used in the tests. Additionally, when compared to FRAP, copper has a faster reaction kinetics. Concerning ABTS and DPPH, CUPRAC has more accessible and stable reagents (110).

The values obtained for the CUPRAC test of the works in this review are shown in Table 05.

Table 5 | Results of the CUPRAC test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Results
Bhuyan *et al*., 2021 (52)	Percentage of inhibition: 21.91 – 95.60% (4.19 – 268.30 µg/mL) IC_{50}: 4.84 – 14.21 µg/mL
Dărăban *et al*., 2019 (64)	123.78 – 357.21 µmol Trolox/mL
Masek *et al*., 2019 (98)	About 0.40 -1.8 (absorbance difference)
Ozdal *et al*., 2018 (110)	1184.94 mg Trolox/g
Ozdal *et al*., 2019 (111)	24.62 – 85.80 mg Trolox/g
Özkök *et al*., 2021 (23)	95.35 – 710.43 mg Trolox/g
Saral *et al*., 2019 (126)	270.00 – 400.00 µmol Trolox/g

ABTS ([2,29-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)])

ABTS assay is based on the decolorization reaction, in which a radical in a stable form is produced before meeting an antioxidant (1,21). Generally, the standard used is Trolox, and the results are expressed as a value of TEAC (Trolox Equivalent Antioxidant Capacity) (162). It has as an advantage reliable results both for fat-soluble and water-soluble antioxidants (1) and for pure substances, mixtures, and plant extracts (29). Furthermore, it maintains its stability over a wide pH range (163), it is fast, cheap, and sensitive (1).

Interestingly, the ABTS test radical is more reactive than the DPPH test. Therefore, their values tend to be more representative of the real antioxidant power of the sample (12,26). This fact occurs because ABTS manages to solubilize itself in both aqueous and oily media, reaching a greater number of targets than DPPH, which is usually present in organic media (138).

The data from the literature review materials for this test are presented in Table 06. Corroborating with other methodologies, the *A. mellifera* propolis has shown good antioxidant activity. For example, the article produced by Salas *et al*. (2016), with similar value to pure standards (164).

Table 6 | Results of the ABTS test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Activity Percentage (%)	Other results
Andrade *et al*., 2017 (43)	-	1868.45 – 2913.55 µmol Trolox/g
Andrade *et al*., 2018 (36)	-	10623.48 – 15106.07 µmol Trolox/g
Augusto-Obara *et al*., 2019 (48)	-	8052.00 – 8773.60 µmol Trolox/g
Béji-Srairi *et al*., 2020 (50)	-	IC_{50}: 244.00 – 616.00 µg/mL
Bhuyan *et al*., 2021 (52)	4.52 - 98.74% (0.78 to 50.00 µg/mL)	IC_{50}: 5.60 – 7.77 µg/mL
Boulechfar *et al*., 2019 (165)	Essential oil: 27.51% MEP: 92.03%	Essential oil IC_{50}: 516.05 µg/mL MEP IC_{50}: 10.08 µg/mL
Calegari *et al.,* 2017 (3)	-	2013: 25.50 – 109.20 µmol Trolox/g 2015: 298.10 – 439.20 µmol Trolox/g
Calegari, 2018 (26)	-	1130.00 µmol Trolox/g
Cao *et al.,* 2017 (7)	-	IC_{50}: 110.28 µg/mL
Cavalaro, 2017[2] (29)	-	408.66 – 13412.14 µmol Trolox/g
Cruz, 2011 (61)	-	46.29 mg of equivalents of gallic acid/g
Da Cunha, 2017 (166)	-	253.56 µmol Trolox/mL
Da Graça Miguel *et al*., 2014 (63)	-	IC_{50}: 9.00 – 1009.00 µg/mL
Dărăban *et al*., 2019 (64)	-	IC_{50}: 0.59 – 0.83 µL
Da Silva *et al*., 2018 (66)	-	19.03 – 1077.00 µmol Trolox/g
De Francisco *et al*., 2018 (30)	-	IC_{50}: 2286.16 µg/mL
De Lima *et al.,* 2019a (25)	-	IC_{50}: 30.10 µg/mL
Del Río Del Rosal *et al*., 2017 (167)	-	16.66 – 63.45 µmol Trolox/g
De Morais *et al*., 2021 (154)	-	2700.00- 35508.8 µmol Trolox/g
De Oliveira, and Andolfatto, 2014 (70)	-	52.08 µmol Trolox/g

Ding *et al*., 2021 (73)	-	36.76 – 106.73 µmol Trolox/g
El Meniiy *et al*., 2021 (77)	-	IC_{50}: 26.00 – 1529.00 µg/mL
Fangio *et al*., 2019 (80)	-	843.00 – 1683.00 µmol Trolox/g
Ferreira, 2017 (6)	-	About 10.00 -100.00 µmol of equivalents of ascorbic acid/g
Gargouri *et al*., 2019 (168)	-	109.76 – 252.90 µmol Trolox/g
Ikeda, 2020 (87)	-	About EEP: 3.12 µmol Trolox/mg Essential oil: 0.40 µmol Trolox/mg
Irigoiti, Yamul, and Navarro, 2021 (88)	-	1.00 - 3.00 µmol Trolox/g
Jansen, 2015 (40)	-	2.45 - 18.00 µg Trolox/mL
Kasote *et al.,* 2017 (12)	-	680.00 – 429.00 µmol Trolox/mg
Kumazawa *et al*., 2013 (21)	23.00 – 71.20%	-
Kumul *et al*., 2020 (92)	-	6310.00 – 64290.00 µmol Trolox/g
Kurek-Górecka *et al*., 2012 (94)	-	139380.00- 153520.00 µmol Trolox
Lacerda, 2012 (35)	-	1.01 – 384.60 mg Trolox/g
Machado *et al*., 2016 (96)	EEP: 77.90 – 98.50% Supercritical: 49.60 – 87.60%	-
Masek *et al*., 2019 (98)	About 35.00 – 89.00% (0.50 – 3.00 mg/mL)	-
Miguel *et al*., 2014 (101)	Winter: 30.18 – 78.90% Spring: 24.77 – 64.76%	-
Mohdaly *et al*., 2015 (102)	94.34% (25.00 µg/mL)	-
Naik, and Vaidya, 2011 (105)	-	420.00 – 5150.00 µmol Trolox (0.10 to 2.00 mg/mL) IC_{50}: 180 µg/mL
Nina *et al*., 2015 (106)	-	870.64 – 2328.66 µmol Trolox/g
Nina *et al*., 2016 (107)	-	225.43 – 2666.56 µmol Trolox/g
Oldoni *et al*., 2015 (109)	-	95.88 µmol Trolox/g
Osés *et al*., 2016 (169)	-	1184.66 -1400.86 µmol Trolox/g
OSÉS *et al*., 2020 (170)	-	280.00 – 470.00 µmol Trolox/g
Ozdal *et al*., 2018 (110)	-	422.82 mg Trolox/g
Pandolfo, 2014 (1)	-	120.00 – 2400.00 µmol Trolox/g
Prasniewski, 2015 (114)	-	22.49 – 354.40 µmol Trolox/g
Quintino *et al*., 2020 (115)	-	IC_{50}: 32.18 µg/mL
Ramnath; Venkataramegowda, 2016 (116)	-	IC_{50}: 298.86 – 860.32 µg/mL
Rodríguez *et al*., 2012 (122)	-	739.70 – 1918.40 µmol Trolox/g

Salas *et al*. 2016 (164)	-	IC_{50}: 14.00 µg/mL
Salas *et al*., 2018 (171)	-	IC_{50}: 23.00 – 30.00 µg/mL
Salas *et al*., 2020 (172)	-	IC_{50}: 29.50 – 33.70 µg/mL
Salgueiro, 2016 (37)	-	EEP: 848600.00 – 1576200.00 µmol Trolox/g MEP: 946300.00 – 1163300.00 µmol Trolox/g Soxhlet: 747800.00 – 2139000.00 µmol Trolox/g
Salgueiro, and Castro, 2016 (124)	-	848600.00 – 1576200.00 µmol Trolox/g
Shehata *et al*., 2020 (130)	About 29.00 - 90.00% (1.00 mg/mL)	-
Shubharani, Mahesh, and Yogananda Murti, 2019 (131)	-	IC_{50}: 15.10 – 550.00 µg/mL
Sousa *et al*., 2019 (134)	-	677600.00 -1068200.00 µmol Trolox/g
Sun *et al*., 2015 (136)	-	IC_{50} WEP: 10310.00 µg/mL IC_{50} EEP: 520.00 – 5520.00 µg/mL
Tiveron, 2015 (27)	-	1.01 – 384.62 mg Trolox/g
Tiveron *et al*., 2016 (138)	-	0.29 – 1.05 µmol Trolox/g
Touzani *et al*., 2018 (140)	-	IC_{50}: 21.00 – 983.00 µg/mL
Yurteri, 2015 (149)	-	0.06 – 2.97 µmol Trolox/g (10.00 – 50.00 µg/mL
Zhang *et al*., 2015 (151)	-	IC_{50}: 40.50 µg/mL
Zhang *et al*., 2016 (152)	-	IC_{50}: 20.00 – 40.50 µg/mL

EEP: Ethanol Extract of Propolis; WEP: Water Extract of Propolis; MEP: Methanol Extract of Propolis

β -Carotene/Linoleic Acid

This system is based on the ability to protect a sample against free radical peroxidative degradation of the lipid substrate linoleic acid (35,37). It is widely used, as it is simple and sensitive, including to analyze extracts of plants (90), as β-carotene is very susceptible to free radicals in the environment (16), in addition to not involving the use of higher temperatures, preventing the degradation of thermosensitive compounds (37).

The analysis of lipid peroxidation is often used due to the sensory and nutritional changes it can cause in food and beverages and the lost nutritional factor, which leads to food waste (29,173). Lipid peroxidation also affects cellular levels; plasma membranes are especially sensitive to ROS attack, promoting changes in their permeability and can even lead to cell death due to the extravasation of cytotoxic enzymes (174).

Second Isla *et al*. (2009), one of the typical compounds of propolis that may be involved in the performance of this plant material in this test is galangin, so synergistic with the other components (175). Generally, results are expressed as IC_{50} (90) or are compared to the Trolox standard (37).

Other results of the review materials are listed below in Table 07.

Table 7 | Results of the β-carotene/linoleic acid system test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Inhibition Percentage (%)	Other results
Béji-Srairi *et al*., 2020 (50)	-	IC_{50}:1300.00 – 2000.00 µg/mL
Boulechfar *et al*., 2019 (165)	Essential oil: 48.51 % MEP: 91.98%	Essential oil - IC_{50}: 198.01 µg/mL MEP - IC_{50}: 43.46 µg/mL
Ceylan, and Halime, 2020 (57)	39.21 – 91.10%	-
De-Melo *et al*., 2014 (16)	51.33 – 92.70 %	-
De Souza *et al*., 2018 (71)	75.50%	-
Ferreira, 2015 (2)	16.86 – 54.25% (40.00 µg/mL); 21.92 – 66.30% (80.00 µg/mL); 15.92 – 75.09% (120.00 µg/mL)	-
Ferreira, 2019 (82)	-	IC_{50}: 101.10 and 106.40 µg/mL
Ferreira *et al*., 2017 (83)	-	IC_{50}: 101.10 and 106.40 µg/mL
Jug, Končić, and Kosalec, 2014 (90)	-	IC_{50}: 14.20 – 55.50 µg/mL
Kurek-Górecka *et al*., 2012 (94)	69.64 and 70.30%	-
Righi *et al*., 2011 (117)	84.50% (1.00 mg/mL); 85.30% (1.50 mg/mL); 85.70% (2.00 mg/mL)	-
Salas *et al*, 2016 (164)	-	IC_{50}: 26.00 and 29.00 µg/mL
Shahbaz *et al*., 2021 (129)	WEP: 39.21% EEP: 49.94 – 60.59% MEP: 48.70 – 57.01%	-

EEP: Ethanol Extract of Propolis; WEP: Water Extract of Propolis; MEP: Methanol Extract of Propolis

Other Forms of Lipid Peroxidation Analysis

There are also other methods able to assess lipid peroxidation. This diversity is due to the great importance of lipid degradation reactions in human health and, especially, in the food industry. One of the common tests used to evaluate lipid peroxidation is the TBARS (Thiobarbituric acid reactive substances), which measures mostly the MDA (malondialdehyde). However, this methodology can also evaluate other volatile substances produced in the lipid oxidation processes (28). Results can be expressed in TBA units: weight in mg of MDA per kg of the sample (174); or even, in Antioxidant Index (AI) – a percentage calculated according to the following formula: IA (%) = 1 – (Sample Abs/Control Abs) x 100, where “Abs” refers to the absorbance obtained (176).

Another methodology used to study lipid peroxidation is the Rancimat method. This test is based on the difference of electrical conductivity of distilled water after the formation of volatile molecules produced by lipid peroxidation reactions, under the accelerated conditions of the method, such as high

temperatures (110 - 130°C) and the presence of oxygen-rich air current (102). Generally, the oxidative stability of the medium is assessed over a period under standard conditions (35). In some cases, the results can be expressed as a "protection factor", in which higher values above one indicates inhibition of lipid peroxidation (178).

The iron thiocyanate methodology is based on quantifying peroxides produced in the initial stages of lipid peroxidation. These substances react in the medium, forming ferric ions that unite with the ammonium thiocyanate and produce the red thiocyanate iron, which devices can detect (132). Still, regarding lipid peroxidation, LDL (Low-Density Lipoprotein) oxidation can be evaluated. It is currently known that this process is one of the first steps to develop some diseases, such as atherosclerosis (155).

Among the different substances that make up the complex propolis, phenolic compounds are responsible for their biological activity. About inhibition of lipid peroxidation, the same turns out to be true. The lipophilic groups of phenolic compounds interact with plasma membrane molecules, preventing their damage. Flavonoids also play an important role in this regard, emphasizing quercetin and rutin, which were shown to be more efficient in inhibiting MDA formation than hesperetin and naringenin, due to their great ability to interact with the membrane phospholipids and the possibility of donating hydrogen atoms, present in their hydroxyl groups (141).

The results found in the works of this literature review for lipid assessment methodologies, are shown below, in Table 08.

Table 8 | Results of other methodologies to assess lipid peroxidation of propolis extracts from *Apis mellifera* obtained from the materials chosen for this literature review.

Author	Test methodology	Results
Boufadi et al., 2014 (53)	TBARS	Peroxidation Inhibition percentage (about): 25.00 – 82.00% (100.00 µg/mL)
Boufadi et al., 2014 (53)	LDL	Inhibition percentage: 77.00 – 99.00% (20.00 µg/mL)
Cavalaro, 2017[2](29)	Iron thiocyanate	At the end of the forced lipid peroxidation process, the propolis emulsion maintained the hydroperoxide levels at the same values as the controls with synthetic antioxidants.
Ceylan, and Halime, 2020 (57)	Iron thiocyanate	Inhibition percentage: 34.74 – 51.77%
Da Graça Miguel et al., 2014 (63)	TBARS	IC_{50}: 14.00 – 699.00 µg/mL
Graikou et al., 2016 (178)	Rancimat	Activities ranged from >1, =1 and <1 (protection factor)
Jeong et al., 2012 (179)	TBARS	IC_{50}: 35.65 µg/mL
Mello, and Hubinger, 2012 (99)	Iron thiocyanate	Decreased absorbance indicates the strong antioxidant activity of propolis extracts.
Mohdaly et al., 2015 (102)	Rancimat	Stabilization factor: 13.7
Osés et al., 2020 (170)	TBARS	0.099 – 0.117 mmol of equivalents of uric acid/g
Permana et al., 2020 (113)	Iron thiocyanate	IC_{50}: 65.32 – 1503.00 µg/mL

Shehata et al., 2020 (130)	TBARS	About 25.00 – 90.00% (1.00 mg/mL)
Silva et al., 2011 (155)	LDL	Propolis samples with higher polyphenol concentrations were able to inhibit lipid oxidation.
Silva et al., 2018 (132)	Iron thiocyanate	>86.00% (5.00 – 100.00 µg/mL)
Tiveron, 2015 (27)	Lipid peroxidation by peroxide index	Propolis extracts (100.00 mg/kg) had good protection against soybean oil oxidation (1.70 – 4.90 mmol O_2/kg) when compared to the control (5.52 mmol O_2/kg).
Xavier et al., 2017 (147)	Lipid peroxidation	60.00 - 90.00% protection for lipid membranes

Food Tests

Rancidity analysis helps ensure the quality and safety of food products and the choice ofproducts by the customer, reducing waste (28).

Alves (2018) studied the insertion of microcapsules of ethanolic extract of propolis (2.00 g) into cakes. The antioxidant activity of the ready food was tested by the DPPH method, with results that ranged from 10.32 - 11.71% inhibition. Such values were higher than those found in cakes with the addition of liquid extract (5.26%), demonstrating that the encapsulation protected the bioactive compounds from the oven temperature. Furthermore, research using a sensory panel showed that the encapsulation caused the organoleptic parameters of the cake were not significantly different from the control, which did not show the addition of extracts (5).

Some works that make up the collection of this review evaluated the protection of propolis extract in salami. These meat products have a large amount of fat, which is responsible for many of their pleasant characteristics, such as juiciness and flavor (28).

Kunrath, and Savoldi (2014) and Kunrath *et al*. (2017) studied how the addition of ethanolic extracts of propolis, atomized by a spray dryer, influences the peroxidation lipid from Italian salami stored for 35 days. The results of the TBARS test showed that the presence of the extract (0.01% and 0.05%) reduced the amount of malondialdehyde present in salami (0.46 and 0.22 mg of MDA/kg) when compared to non-treated ones (0.51 mg of MDA/kg). Also, the sensory panel analysis demonstrated that the salami that received the propolis did not differ significantly in sensory parameters compared to the control salami (28,93).

Vargas-Sánchez *et al*. (2019) opted to study the antioxidant activity of propolis in extracts from beef and pork steaks. When compared to the control group, samples that received the extract of propolis had an 88.70% decrease in MDA production by the TBARS method. In addition, this natural product reduced the protein oxidation of the meat sample (141).

Electrochemical Techniques: Voltammetry

The reducing power of a substance can be characterized by its oxidizing potential, both of which are inversely proportional. One way to evaluate the power reducer of antioxidant substances is using electrochemical techniques (180). Such methodologies prove to be useful not only to obtain detailed information about the antioxidant potential but also to observe the number of electrons involved in the process, reaction mechanisms, process reversibility, and equilibrium constants. The most popular electrochemical techniques are differential pulse voltammetry, with high resolution and accuracy, and cyclic voltammetry (98,181).

Through the cyclic voltammetry technique, the oxidative process of a substance is characterized by the generation of a potential difference between the electrodes of the electrochemical cell (work and reference). Then, the anodic peak potential (Ep) and the current intensity magnitude (Ip) are analyzed. The Ep can be associated with the information of electronic transfer energy, providing data on a substance's antioxidant potential. The Ip parameter also predicts an estimate of the antioxidant capacity relating to the electrical charge potentially transferred (181,182).

The differential pulse technique is based on measuring the current immediately after a pulse is released, as well as at the end of it. It uses a solid electrode, which reduces adsorption problems and increases test sensitivity (58).

One way of expressing results is in TEAP (Total Electrochemical Antioxidant Power). This value corresponds to the sum of the current density of each electrochemical process value, obtained at the maximum peak (58).

The fact that they can be used in the analysis of isolated substances or complex extracts and being sensitive, selective, and reproducible, even for samples from biological matrices can be mentioned as an advantage of this methodology. Furthermore, they allow the analysis of antioxidant molecules without the need for the use of additional reagents, being quick and simple processes. However, it is important to emphasize that, concurrently with electrochemical processes, and other chemical reactions may also occur. For this reason, the correlation of parameters of this technique is not always perfectly correct (13,181). The values obtained for the voltametric techniques of the present literature review are shown in Table 09.

Table 9 | Results of the voltammetry test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Results
Coelho, 2013 (58)	0.06 – 0.49 (electrochemical antioxidant power – 0.40 V) or 0.06 - 0.60 mg of p-cumaric acid/mL
Coelho *et al*., 2017 (20)	0.06 – 0.49 (electrochemical antioxidant power – 0.40 V) or 0.06 - 0.60 mg of p-cumaric acid/mL
De Oliveira, 2015 (182)	19.00 – 54.00 mmol of equivalents of galic acid (Ipa)
Falcão, 2013[4](13)	0.14 – 1.14 V or 9.00 – 73.00 TEAP
Masek *et al*., 2019 (98)	1.12 – 1.39 V

Metal Chelation

The role of transition metals in oxidative stress is already well-established. Among them, cobalt, vanadium, arsenic, nickel, and chromium can be mentioned. Also, iron and copper stand out, capable of promoting the production of hydroxyl radicals through the Fenton reaction. There is the interaction of reduced metallic ions with hydrogen peroxide, giving rise to free radicals and oxidized metallic ions. The products of this reaction are capable of degrading cell plasma membranes through oxidative attack to the abundant polyunsaturated fatty acids abundantly present. Also, metals can be electron donors or act as catalysts for chemical reactions (90,183).

Substances able to chelate these ions and thus prevent oxidative damage to biological molecules are classified as secondary antioxidants (Figure 03). By making them less available in the medium, their oxidative potential is reduced, stabilizing them, and reducing the likelihood of interacting with molecular targets (90).

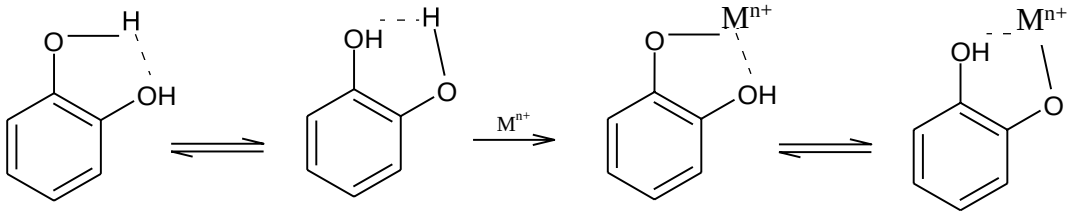

Figure 03 | Schematic process of the chelation mechanism for metals. Source: Adapted from Salgueiro, 2016 (37).

Propolis has compounds capable of helping in this picture described through the chelation of metals in the medium. For example, apigenin, naringenin, and diosmin (101). Through electrochemical measurements, other substances, such as rutin and catechin, have been described as important zinc and copper chelators (181).

Due to its high reactivity, iron is known as a direct and indirect inducer of lipid peroxidation. Due to its great importance in oxidative stress and damage to cell structures, iron is one of the most studied by researchers (157). In this review, all studies evaluated the power of propolis samples in sequestering the iron ion. The results are shown in Table 10.

Table 10 | Results of the iron chelation test of *Apis mellifera* propolis extracts obtained from the materials

chosen for this literature review.

Author	Results
Abubaker, and Fageer, 2017 (157)	8.73 – 43.25% (125.00 – 1000.00 µg/ml)
Jug, Končić, and Kosalec, 2014 (90)	IC_{50}: 324.90 – 1840.40 µg/mL
Miguel *et al*., 2014 (101)	Winter: 43.92 – 82.35% Spring: 41.11 – 71.34%
Wali *et al*., 2016 (144)	IC_{50}: 74.94 – 136.88 µg/mL

Enzymatic Activity

The organism naturally has mechanisms to control oxidizing agents, such as degradation enzymes (catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, glutathione S-transferase, among others), and different support molecules, such as reduced glutathione, a cofactor for enzymes, which has thiol groups responsible for scavenging free radicals; if such molecules fail to produce or are inhibited, there is a decrease in the redox potential of the cells and the organism enters oxidative stress, leading to cell damage (184), mitochondrial damage, caspase activation and apoptosis (4).

One of the first lines of antioxidant defense is the metalloenzyme superoxide dismutase (SOD), whose function is to catalyze the transformation of the superoxide radical into hydrogen peroxide or oxygen. Catalase (CAT), in turn, is present in cellular peroxisomes and is responsible for breaking two hydrogen peroxide molecules into molecular oxygen and water, in a reaction of dismutation (184,185).

Glutathione reductase manages to eliminate several harmful compounds, such as singlet oxygen, hydroxyl radicals, and several other electrophiles. Glutathione peroxidase can convert hydroperoxyls and hydrogen peroxide to water (186).

Myeloperoxidase (MPO) is contrary to the enzymes mentioned so far. It is found in neutrophil granules, responsible for catalyzing the reaction between chloride and hydrogen peroxide ions, with the production of hypochlorous acid. This acid plays a crucial role in the destruction of invading pathogens. However, when found in extracellular tissues, or cases of exacerbation of the immune response and chronic diseases, with the so-called “neutrophil net”, there may be an excess of free radicals in the medium, causing damage to various biomolecules (53).

The function of xanthine oxidase is to produce uric acid, with lipoxanthine and xanthine as substrates. After this process, the enzyme is reoxidized, at which point molecular oxygen acts as an electron acceptor, culminating in the production of hydrogen peroxide and superoxide radicals. The lipoxygenase enzyme (LOX) is also noteworthy, essential in the emergence of inflammatory processes and playing a role in oxidative phenomena, with the catalysis of unsaturated acids oxidation reactions. It is responsible for producing inflammatory leukotrienes from arachidonic acid. Furthermore, it promotes the conversion of linoleic acid to 13-hydroperoxide linoleic acid, which can be quantified by a spectrophotometer at 234 nm (63,187).

Due to the great importance that such catalytic biomolecules develop in protecting against oxidative stress, some works strive to verify the activity of these enzymes. In some cases, it is interesting that the enzymes are overactive or over-expressed, which helps the body deal with free radicals such as SOD and catalase. Moreover, in others, enzyme inhibition is more advantageous if the enzyme is a producer of oxidizing species or has a role in some of the cellular damage pathways and tissue, such as MPO.

The propolis compounds generally associated with antioxidant activity with an enzyme modulating mechanism are phenolic compounds and flavonoids, such as galangin. LOX is inhibited by CAPE (caffeic acid phenethyl ester), caffeic acid, quercetin, and naringenin molecules (187).

The results of enzymatic activity after the addition of the propolis extract are listed below, in Table 11. Generally, they were expressed as IC_{50} in the case of enzymes harmful to the organism.

Table 11 | Results of the test to assess the enzymatic modulation of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Test methodology	Results

Boufadi *et al*., 2014 (53)	Inhibition of MPO	IC_{50}: 10.30 – 48.70 µg/mL
Da Graça Miguel *et al*., 2014 (63)	Inhibition of LOX	IC_{50}: 149.00 – 2521.00 µg/mL
El-Guendouz *et al*., 2016 (187)	Inhibition of LOX	IC_{50}: 20.00 – 653.00 µg/mL
El-Guendouz *et al*., 2016 (187)	Inhibition of xanthine oxidase	IC_{50}: 8.00 – 3116.00 µg/mL
Salas *et al*, 2016 (164)	Inhibition of cyclooxygenase 2	IC_{50}: 100.00 and 106.00 µg/mL
Salas *et al*, 2016 (164)	Inhibition of LOX	IC_{50}: 63.90 and 94.90 µg/mL
Salas *et al*., 2018 (171)	Inhibition of LOX	IC_{50}: 90.00 – 100.00 µg/mL
Shubharani, Mahesh, and Yogananda Murti, 2019 (131)	Activity of SOD	IC_{50}: 21.65 – 25.91 µg/mL
Silva *et al*., 2011 (155)	eNOS	Propolis samples increased eNOS expression
Silva *et al*., 2011 (155)	NADPH oxidase	Propolis samples decreased NADPH oxidase activity

ROS and RNS Scavenging

Among the most famous free radicals studied, there are the so-called ROS, or Reactive Oxygen Species, which can be mentioned the hydroxyl radical ($^{\bullet}OH$) the most reactive of them, the superoxide radical ($O2^{\bullet-}$) and hydroperoxyl ($ROO^{\bullet}$). Such compounds can quickly interact with the nitrogenous bases of the DNA, causing mutations, single or double-strand breaks, nitrogenous base modifications, and cross-linking. Furthermore, damage to proteins and lipids, causing poor folding and lipid peroxidation, respectively, are reported (9,90).

The superoxide radical can cross several cell barriers, being transported by anionic channels, and, physiologically, it is thought to be responsible for mediating nerve signals, not being very reactive. The same applies to hydrogen peroxide, produced by enzymes called peroxidases, from oxygen metabolism. It is very soluble and inert, with low oxidizing power; however, this one can produce new free radicals when penetrating cells and reacting with iron or copper ions. Finally, the most reactive oxidizing agent is the hydroxyl radical. It manages to have rapid formation and dissemination through reactions in the body (1,144,157).

Other molecules are important in the context of oxidative attack: singlet oxygen, hypochlorous acid radical - very linked to chronic inflammatory diseases and lipid peroxidation, and molecules from nitrogen, such as nitric oxide (NO), a fat-soluble gaseous radical capable of crossing biological membranes, and the peroxynitrite ($ONOO^{\bullet-}$), a potent vasodilator. Reactive nitrogen species (RNS) are associated with several diseases, including atherosclerosis, cancer, diabetes, and neurodegenerative disorders (1,30).

Regarding damage to the central nervous system, the role of nitric oxide is noted, when reacting with the superoxide radical, producing peroxynitrites. This product is harmful to neurons leading to cell apoptosis (188). Some antioxidant compounds present in propolis extracts compete with oxygen, preventing its reaction with nitric oxide and, consequently, the formation of toxic nitrites and RNS (116).

Several methodologies observe the decrease of free radicals from the reaction medium. Some of them use the Fenton reaction (73), while others observe the decrease in absorbance (144). The superoxide anion measurement assay uses the latter mechanism, by analyzing the decrease in absorbance after reaction between nitrotetrazolium blue and free radicals, generating formazan salt, which can be perceived at 560 nm (101,112). The results of these tests are presented in Table 12.

Table 12 | Results of the ROS and RNS sequestration assessment test of *Apis mellifera* propolis extracts obtained from the materials chosen for this literature review.

Author	Radical	Results
Abubaker, and Fageer, 2017 (157)	Hydrogen peroxide	60.37 – 92.68% (125.00 to 1000.00 µg/mL)
Da Graça Miguel *et al*., 2014 (63)	Hydroxyl radical	IC_{50}: 59.00 – 1389.00 µg/mL
De Francisco *et al*., 2018 (30)	Nitric oxide	IC_{50}: 1.61 µg/mL
De Francisco *et al*., 2018 (30)	Superoxide	IC_{50}: 226.778 µg/mL
De Francisco *et al*., 2018 (30)	Hydrogen peroxide	IC_{50}: 520.29 µg/mL
De Francisco *et al*., 2018 (30)	Hypochlorous acid	IC_{50}: 13.25 µg/mL
De Francisco *et al*., 2018 (30)	Peroxyl radical	IC_{50}: Did not reach IC_{50}
Ding *et al*., 2021 (73)	Hydroxyl radical	60.00 – 77.00% (5.00 mg/mL)
Ding *et al*., 2021 (73)	Superoxide	52.00 – 63.00% (5.00 mg/mL)
Fangio *et al*., 2019 (80)	Hydroxyl radical	0.34 nmol of scavenged hydroxyl radicals (0.04 ng of propolis extract)
Gargouri *et al*., 2019 (168)	Hydroxyl radical	0.052 – 0.068 mmol of equivalents of uric acid/g
Miguel *et al*., 2014 (101)	Superoxide	Winter: 46.02 – 85.72% Spring: 48.18 – 84.79%
Naik, and Vaidya, 2011 (105)	Nitric oxide	5.15 – 53.18% (0.10 – 2.00 mg/mL) IC_{50}: 1650.00 µg/mL
Naik, and Vaidya, 2011 (105)	Superoxide	0.834 – 1.457 nmol Trolox/g
Osés *et al*., 2016 (169)	Hydroxyl radical	0.0012 mmol of equivalents of uric acid/mL
OSÉS *et al*., 2020 (170)	Superoxide	IC_{50}: 20.00 – 440.00 µg/mL
Pazin *et al*., 2017 (112)	Superoxide	IC_{50}: 34.00 µg/mL
Ramnath, and Venkataramegowda, 2016 (116)	Nitric oxide	IC_{50}: 536.19 – 757.75 µg/mL
Ramnath, and Venkataramegowda, 2016 (116)	Hydrogen peroxide	IC_{50}: 325.30 – 765.75 µg/mL
Salas *et al*., 2020 (172)	Hydroxyl radical	IC_{50}: 16.50 – 37.00 µg/mL
Salas *et al*., 2020 (172)	Superoxide	IC_{50}:115.00 – 290.00 µg/mL
Salas *et al*., 2020 (172)	Hydrogen peroxide	IC_{50}: 39.00 – 92.00 µg/mL
Salas *et al*., 2020 (172)	Nitric oxide	IC_{50}: 50.00 – 104.50 µg/mL

Schmidt *et al*., 2014b (128)	Anti-radical activity – Hydrogen peroxide	0.48 – 2.94 mg Trolox/L (2.9×10^{-5} g/mL)
Tiveron, 2015 (27)	Superoxide	IC_{50}: 0.29 – 2.91 µg/mL
Tiveron, 2015 (27)	Hypochlorous acid	IC_{50}: 0.03 – 1.45 µg/mL
Tiveron *et al*., 2016 (138)	Hypochlorous acid	IC_{50}: 0.03 – 1.45 µg/mL
Wali *et al*., 2016 (144)	Hydrogen peroxide	IC_{50}: 109.93 – 145.42 µg/mL
Yuan *et al*., 2020 (148)	Hydroxyl radical	IC_{50}: 54.42 – 59.61 µg/mL
Zhang *et al*., 2015 (151)	Superoxide	1,52 mmol Trolox/g
Zhang *et al*., 2016 (152)	Superoxide	0.96 – 1.65 mmol Trolox/g

Cell Culture Tests

Some authors of the materials in this literature review chose to use cellular assays, among others, to measure the antioxidant activity of extracts in a little more complex environment, which is more similar to reality. It is known that free radicals can be generated by reactions in both the intracellular and extracellular environments. The cells most recognized as producers of such oxidizing species are those that make up the immune system (neutrophils, monocytes, eosinophils, and macrophages), in addition to endothelial cells (44).

One of the methodologies used to measure the antioxidant potential of the ethanol extract of propolis, used by Cao *et al*. (2017), was the evaluation of ROS production by macrophages, *in vitro*, when stimulated by hydrogen peroxide (Fenton reaction). Propolis was able to inhibit the production of ROS in a concentration-dependent manner and increase the survival of these cells (7). Similar tests have been used by Sun *et al*. (2015), who assessed the antioxidant capacity of propolis extracts in HepG2 cells (cellular antioxidant activity). The IC_{50} found ranged from 171.00 - 25738.00 µg/mL, depending on the proportion of water and ethanol in the solvent (136). Gokduman (2019) also worked with HepG2 cells and Hep3B. Treatment with ethanol extract of propolis (10.00 – 100.00 µg/mL) significantly reduced intracellular ROS production (159).

The paper proposed by Bonamigo *et al*. (2017) brought the MDA dosage test, a marker of oxidative damage to membrane lipids. For that purpose, erythrocytes, oxidation inducers, and markers of the desired molecule were used. Afterward, the absorbance of the sample of supernatants was read at 532 nm. As a result, it was noticed that the ascorbic acid control and the propolis sample reduced MDA levels by 65.70% and 38.40%, respectively, at the highest concentration evaluated. The same work also evaluated the antioxidant activity through the inhibition of oxidative hemolysis: the ethanol extract obtained an inhibition of 24,60%, at a concentration of 125.00 µg/mL (4).

Valent *et al*. (2011) also evaluated the protection of the methanolic extract of Portuguese propolis in protecting red blood cells against oxidative lysis processes. As expected, treatment with propolis decreased MDA production levels (71.00% to 78.00%), due to reduced lipid peroxidation reactions in cell membranes, when compared to control groups (IC_{50}: 8.10 and 9.70 µg/mL). In addition, propolis protected cells against hemolysis caused by damage to the membranes, as free radicals disturb surface structures and ion channels (IC_{50}: 6.30 and 10.70 µg/mL) (189).

Working with human breast cancer cells (MCF-7), Arslan *et al*. (2021) measured lipid peroxidation (TBARS) when exposed to mitomycin C. Treatment with propolis extracts (32.50; 65.00; 125.00; 250.00 and 500.00 µg/mL) reduced the levels of lipid peroxidation (0.03 – 0.16 nmol/mL), when compared to untreated control groups (0.40 nmol/mL) (47).

Salas *et al*. (2016) studied the effects of pretreatment with ethanolic extract of propolis on macrophages stimulated with lipopolysaccharide (LPS), a potent pro-inflammatory molecule. It was noted that the treated group had a decrease in the levels of NO production (IC_{50}: 8.40 and 9.40 µg/mL) and its inducible enzyme (iNOS) (IC_{50}: 27.03 and 30.96 µg/mL) (164). The same strategy was used by Bhuyan*et al*. (2021). Propolis treatment also decreased NO production by macrophages, with IC_{50} ranging from2.06 - 22.5 µg/mL (52).

Salgueiro (2016) tested the protective activity of the ethanol extract of propolis against damage to the yeast plasma membrane caused by hydrogen peroxide. The results of the TBARS method verified that

the presence of the treatment with propolis reduced the oxidative damage in the membranes (37).

Also working with yeasts, Cruz (2011) used these microorganisms to study the protective effect of propolis against oxidative damage to DNA and its viability against oxidative stress promoted by hydrogen peroxide. Exposure to pre-treatment and co-treatment with propolis extracts improved yeast cell viability compared to exclusive hydrogen peroxide controls. However, post-treatment with propolis could not reverse the oxidative damage that had already occurred. Also, at concentrations of 25.00 - 300.00 µg/mL, this natural product managed to protect the DNA from oxidative damage. However, in higher concentrations, it also had pro-oxidative and genotoxic effects (61).

Mendez-Pfeiffer *et al*. (2020) studied the effect of treatment with the methanol extract of propolis on M12.C3 cells. F6, when exposed to an environment of oxidative stress caused by the application of hydrogen peroxide. Treatment with propolis (25.00 and 50.00 µg/mL) increased cell survival and decreased damage to the plasma membrane by inhibiting the excess production of intracellular ROS (100).

Jeong *et al*. (2012) analyzed the protective effect of aqueous propolis extract on central nervous system cell survival after oxidative stress induction by hydrogen peroxide. At 25.00 µg/mL, pre-treatment with propolis had a good protective effect, similar to ascorbic acid (200.00 µmol/L), increasing cell survival compared to stress control. Furthermore, the protection against damage to the membrane of PC12 cells was studied, using the enzyme lactate dehydrogenase as a cell lysis marker. At a 100 µg/mL concentration, the extract managed to decrease the enzyme activity from 257.00% (control) to 145.00%, demonstrating protection against cell extravasation (179).

Zeitoun *et al*. (2019) studied the effects of propolis extract on macrophages (RAW 264.7) exposed to LPS, about NO production, and enzyme expression inflammatory drugs such as cyclooxygenase 2 and iNOs. Treatment with propolis (1.00; 5.00; 10.00; 15.00; 30.00; 40.00; 50.00; and 60.00 µg/mL) demonstrated the inhibition of both enzymes, in a dose-dependent way, obtaining almost complete inhibition at a concentration of 60.00 µg/mL. NO production levels also decreased compared to the control groups (150).

Working with human colon cancer cells (HCT-116), Žižić *et al*. (2013) evaluated the intracellular production of superoxide radicals and nitrite. In the presence of propolis extracts (10.00; 50.00; 100.00 and 500.00 µg/mL), there was a decrease in the level of both molecules 24 and 72 hours after the beginning of the tests (153).

Al Naggar *et al*. (2016) followed a different path by evaluating the transcription of Nrf2 (nuclear erythroid factor 2 related to factor 2), a transcription factor activated in cellular stress cases. In such situations, it migrates to the nucleus and binds to promoters, culminating in the transcription of oxidative stress response genes. The stress environment was provided using hydrogen peroxide. Treatment with propolis extract at a concentration of 25.00 µg/mL promoted inhibition of 2.33 - 22.85% of the activation of these, demonstrating that propolis reduces cell stress and, consequently, it reduces the need to transcribe these genes (42).

Similarly, Zhang *et al*. (2015) and Zhang *et al*. (2016) analyzed the expression of antioxidant genes in macrophages. Exposure to propolis extract (2.50; 10.00 and 15.00 µg/mL) significantly increased the expression of these genes, demonstrating the ability of propolis to induce the synthesis of antioxidant mediators. In addition, it was determined that the treatment with propolis extract could reduce intracellular levels of ROS, through the induction of hydrogen peroxide, to amounts smaller than the initial basal levels, in agreement with the results of other studies (151,152).

In vivo **Tests**

It is known that propolis has low innate toxicity (11), since the main chemical constituents, the flavonoids, also have low toxicity (28). Rodents that consumed a daily dose of 1400 mg/kg of this natural product had no side effects. In cats, 100 mg/kg was well tolerated by subcutaneous injections. Studies have inferred that humans can safely ingest up to 1.4 mg/kg per day (about 70 mg/day) (11,14,166). Given this, propolis is considered safe for consumption, having GRAS (Generally Recognized as Safe) status. However, ingesting large amounts of propolis in its raw state can cause discomfort in the gastrointestinal tract, as well as an increase in cases of contact dermatitis in cosmetic formulations (11).

The work of Capucho *et al*. (2012) presented the evaluation of oxidative stress in the epididymis of rats treated with propolis gavage at a concentration of 3.00; 6.00; and 10.00 mg/kg/day, during 56 days. At the end of the process, animals were sacrificed, and epididymis was prepared and evaluated. Treatment with aqueous propolis extract increased the production of reproductive cells. However, it did not increase the oxidative environment that usually accompanies this growth. There was no difference in the TBARS test values and the CAT enzyme activity, only an increase in the thiol levels compared to the control

group (190).

Cécere *et al*. (2021) evaluated oxidative parameters in the blood of lambs supplemented with propolis extract at concentrations of 150.00; 200.00 and 250.00 µL/kg/day for 42 days. The results for the treatment with 250.00 µL/kg/day, on the 42nd day, showed a decrease in ROS levels (243.24 U DCF/mg protein in the control group and 250.94 in the treated group). Unexpectedly, there was a decrease in SOD activity levels (7.17 U SOD/mg protein in the control group and 6.40 in the treated group) and increased NO expression (4.70 mmol NO/mg protein in the control group and 5.34 in the treated group). However, there was an increase in the levels of thiols from proteins (3.11 mmol SH/mL in the control group and 5.37 in the treated group) and from other sources (4.41 mmol/mL in the control group and 6.55 in the group treated) as well as nitrites/nitrates, widely known for their inflammatory capacity (56).

Yonar *et al*. (2012) also performed tests on hematological parameters, in addition to specific organ analyses; however, their specimens were fish (*Ciprinus carpio carpio*) exposed to the pesticide chlorpyrifos. In the control group that received only the pesticide, the parameters worsened, indicating oxidative stress: increased levels of MDA and SOD activity decreased CAT activity and reduced glutathione activity. Treatment with propolis (10.00 mg propolis/kg body weight) resulted in the parameters reverting to normal levels. However, the treatment with propolis only had no significant effect on test values (184). A similar experiment was carried out by Yonar *et al*. (2014). However, this time, the fishes were exposed to the pesticide malathion. Again, the oxidative stress parameters were altered in the group exposed to this substance, and there was a significant improvement when the propolis treatment was started (levels of MDA, SOD, CAT, reduced glutathione, and glutathione peroxidase) (191).

The same group continued the research line by analyzing the effect of the propolis diet on crayfish and their eggs. This natural material supplementation results in reduced lipid peroxidation, decreased MDA levels, and improved activity of antioxidant enzymes (173).

Da Silveira *et al*. (2016) studied the effect of propolis on behavior and hematological parameters of oxidative stress in Wistar rats, right after stressful tests such as forced swimming and open-field anxiety tests. Treatment with yellow propolis EEP (1.00; 3.00; 10.00; and 30.00 mg/kg) was observed to reduce the production of NO and MDA. Still, there was no change in the level of enzymes such as catalase and superoxide dismutase, induced by stress, nor the ABTS test values (188).

The work carried out by Jeong *et al*. (2012) aimed to analyze the neuroprotective effect of aqueous extracts of propolis in the brain of rats exposed to oxidative stress. The results indicated that the treatment reduced cell death when exposed to hydrogen peroxide and decreased intracellular enzyme release (represented by lactate dehydrogenase), indicating that the plasma membrane was intact in most of the cells (179).

Rivera-Yañez *et al*. (2018) evaluated the enzymatic activity present in the pancreas of rats with a model of diabetes-induced by intraperitoneal injection of streptozotocin (130.00 mg/kg). Treatment with propolis was performed for 15 days at a dose of 0.30 g/kg/day. The colorimetric evaluation methods of the enzymatic activity showed an increase in the activity of the enzymes SOD, CAT, and GPx (glutathione peroxidase) compared to the control groups, demonstrating the ability of propolis to fight pathological oxidative stress even in organ systems (120).

Saleh (2012) studied the protective effects of propolis against hepatotoxicity caused by the administration of 4-tertiary-octylphenol (100.00 mg/kg) during six weeks. This generated a decrease in the enzymatic activity of GST (glutathione S-transferase), SOD, and CAT, demonstrating the role of this substance in causing oxidative stress in the evaluated tissues. Treatment with aqueous extracts of propolis (100.00 mg/kg) increased the level of all enzymes, even above the values found in the control groups that did not receive external stress. One of the possible mechanisms of action of this natural product is the presence of trace mineral compounds, such as zinc, magnesium, manganese, and nickel, responsible for the reactivation of such enzymes. In addition, the group treated with propolis also showed a reduction in MDA levels in hepatocytes, performed using the TBARS test (192).

The work developed by Tohamy *et al*. (2014) analyzed the effect, among other activities, of aqueous extracts of Egyptian propolis on the oxidative status of tissues in rats (*Mus musculus*) exposed to cisplatin (2.80 mg/kg twice a week for 2 weeks). Such animals showed an increase in oxidative stress, due to increased levels of lipid peroxidation (TBARS) and decreased activity of antioxidant factors such as catalase and glutathione. The group receiving the propolis treatment (8.40 mg/kg/day, for 14 days) managed to satisfactorily reverse the peroxidation and return to normal enzyme values (CAT and GSH) (193).

Wiwekowati *et al*. (2020) studied the anti-lipid peroxidation effect using an ELISA kit that analyzes the

levels of MDA existing in the medium. The rats (*Rattus norvegicus*) were subjected to placement a foreign body in the jaw (coil spring), and their blood was removed for analysis purposes. The results showed that groups treated with the propolis gel had reduced MDA values (194).

Other Methodologies

In this session, data from tests less frequently used in the selected works were compiled. The Phosphomolybdenum method is used to evaluate the total antioxidant capacity, evaluating both lipophilic and hydrophilic compounds. It is based on reducing molybdenum VI to V in an acidic environment, with subsequent formation of a complex between this molecule and the phosphate, whose color is greenish. Its advantages are low cost, simplicity, plus the fact that it can be used for complex matrices, such as propolis (45,50).

Another method used is the SNPAC (Silver Nanoparticles Antioxidant Capacity). In the presence of antioxidants, there is a reduction of the silver ion, present in silver nitrate, resulting in nanoparticles suspended in solution, stabilized by trisodium citrate. At that moment, the reaction medium changes color, from pale yellow to brown, being sensed at 423 nm (64). The FOX test (Ferrous Oxidation Xilenol Orange) is a spectrophotometric methodology that relies on the ability of hydroperoxides to oxidize iron from its ferrous to ferric form in an acidic medium. A colored complex with xylenol orange is formed when this occurs, perceived at 560 nm (76).

The results of these tests are shown in Table 13.

Table 13 | Results of the test to evaluate the antioxidant activity of *Apis mellifera* bee propolis extracts by different methods obtained from the materials chosen for this literature review.

Author	Test methodology	Results
Araújo *et al*., 2020 (45)	Phosphomolybdenum	22.72% (inhibition percentage when compared to 200 µg/mL ascorbic acid)
Béji-Srairi *et al*., 2020 (50)	Phosphomolybdenum	158.66 – 220.44 mg of equivalents of gallic acid/g
Dărăban *et al*., 2019 (64)	SNPAC	157.31 – 619.53 µmol Trolox/mL
Duca *et al*., 2019 (76)	FOX	About 18.00 - 32.00% inhibition of hydrogen peroxide (5.00 and 0.50 mg/mL)
El Meniiy *et al*., 2021 (77)	Phosphomolybdenum	6.81 – 80.82 mg of equivalents of ascorbic acid/g
Karadal *et al*., 2018 (195)	Total antioxidant Status (TAS) - ELISA	7.30 – 9.45 µmol Trolox/g
Silva *et al*., 2011 (155)	Inhibition of tyrosine nitration	IC_{50}: 50.00 µg/mL
Touzani *et al*., 2018 (140)	Phosphomolybdenum	6.56 – 90.87 mg of equivalents of ascorbic acid/g

CONCLUSION

Given the numerous biological properties attributed to propolis, such as antimicrobial, antioxidant, anti-inflammatory, and anti-cancer, associated with consumers' search for safe (GRAS) and ecological products, this balsamic material is the subject of several types of research around the world, in order of being used by the food and pharmaceutical industry in new formulations, both as an active ingredient and as an adjuvant.

In this literature review, it can be noticed that propolis samples have extreme variations in terms of antioxidant activity. But, overall, this natural product showed great antioxidant power in all reported tests (both *in vitro* and *in vivo*), through multiple mechanisms, such as ROS and RNS sequestration, metal chelation, and inhibition of pro-oxidant enzymes. As already demonstrated in the literature several times, this variation is due to external factors like the botanical source, seasonality, bee species, collection method, extracting, and testing biological activities.

CONFLICT INTEREST

The authors declare no personal or financial conflict of interest related to this work.

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SUPPLEMENTARY MATERIALS
Authors have provided supplementary materials. Table S1: Search strategy in each database chosen for this literature review. Table S2 | Data on the collection methodology and information on samples of the 173 materials used in this literature review.

AUTHOR CONTRIBUTIONS
All authors contributed to Conceptualization, Methodology, Formal analysis, Investigation, Writing. (Y.M.F.B.) Review & Editing.

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REFERENCES

1. Pandolfo, V. Z. (2014) 'Caracterização e efeito da variação sazonal da própolis orgânica produzida no sul do Brasil', Master thesis, University of São Paulo, Piracicaba. Available in: https://www.teses.usp.br/teses/disponiveis/11/11141/tde-05012015-154728/en.php

2. Ferreira, J. M. (2015) 'Características sensoriais, físico-químicas e atividade biológica da própolis produzida no semiárido do estado do Rio Grande do Norte, Brasil', Masters dissertation, Federal Rural University of the Semiarid Region, Mossoró. Available in: https://repositorio.ufersa.edu.br/handle/tede/378

3. Calegari, M. A. *et al*. (2017) 'Propolis from Southwest of Parana produced by selected bees: Influence of seasonality and food supplementation on antioxidant activity and phenolic profile', *Annals of the Brazilian Academy of Sciences*, 89(1), 45-55. DOI: 10.1590/0001-3765201620160499

4. Bonamigo, T. *et al*. (2017) 'Antioxidant and cytotoxic activity of propolis of Plebeia droryana and *Apis mellifera* (Imenoptera, Apidae) from the Brazilian Cerrado biome', *PloS one*, 12(9). DOI: 10.1371/journal.pone.0183983

5. Alves, C. J. (2018) 'Microencapsulação de própolis utilizando matrizes proteicas para aplicação como ingrediente funcional em alimentos', Doctoral thesis, Federal University of Pelotas, Pelotas. Available in: http://www.guaiaca.ufpel.edu.br/handle/prefix/4131

6. Ferreira, V. U. (2017) 'Caracterização química, atividades antioxidante, antileucêmica e antimicrobiana da própolis âmbar sul brasileira', Masters dissertation, Federal University of Pampa, São Gabriel. Available in: https://dspace.unipampa.edu.br//handle/riu/1467

7. Cao, X. P. *et al*. (2017) 'Mechanisms underlying the wound healing potential of propolis based on its *in vitro* antioxidant activity, *Phytomedicine*, 34, 76-84. DOI: 10.1016/j.phymed.2017.06.001

8. Fianco, A. L. B. *et al*. (2014) 'Estudo sobre a atividade antifúngica e antioxidante de extratos de própolis obtidos com CO2 supercrítico', Masters dissertation, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre. Available in: http://tede2.pucrs.br/tede2/handle/tede/3255

9. Gonçalves, G. M. S., Santos, N. P., and Srebernich, S. M. (2011) 'Antioxidant and antimicrobial activities of propolis and açai *(Euterpe oleracea Mart)* extracts', *Revista de Ciências Farmacêuticas Básica e Aplicada*, 32(3), 349-356.

10. Aguiar, G. R. (2015) 'Estudo químico e avaliação biológica da própolis vermelha de Alagoas', UFC Institutional Repository. Masters dissertation, Federal University of Ceará, Fortaleza. Available in: http://www.repositorio.ufc.br/handle/riufc/16935

11. Wagh, V. D. (2013) 'Propolis: a wonder bees product and its pharmacological potentials', *Advances in pharmacological sciences*, 2013. DOI: 10.1155/2013/308249

12. Kasote, D. M. *et al*. (2017) 'HPLC, NMR based chemical profiling and biological characterization of

Indian propolis', *Fitoterapia*, 22, 52-60. DOI: 10.1016/j.fitote.2017.08.011

13. Falcão, S. I. D. M. (2013) 'Chemical Composition of Portuguese Propolis. Bioactive Properties', Doctoral thesis, University of Porto. Available in: https://www.proquest.com/openview/2dfa8019c2f5172c7b526d924c2bcbdc/1?pq-origsite=gscholar&cbl=2026366

14. Adelmann, J. (2005) 'Própolis: Variabilidade composicional, correlação com a flora e bioatividade antimicrobiana/antioxidante'. Master thesis, Federal University of Paraná, Curitiba. Available in: https://www.acervodigital.ufpr.br/handle/1884/1249

15. Technavio. Propolis Market to grow at a CAGR of 5.41% from 2020 to 2025|Evolving Opportunities with Apiary Polenectar & Apis Flora Industrial e Comercial Ltda.|17000+ Technavio Reports. Available in: https://www.prnewswire.com/news-releases/propolis-market-to-grow-at-a-cagr-of-5-41-from-2020-to-2025evolving-opportunities-with-apiary-polenectar--apis-flora-industrial-e-comercial-ltda17000-technavio-reports-301454553.html. Access 27/01/2022. Last updae 06/01/2022

16. De-Melo, A. A. M. *et al*. (2014) 'Antioxidant activity of propolis', *Pesquisa Agropecuária Tropical*, 44(3), 341-348. doi: 10.1590/S1983-40632014000300004

17. Funari, C. S., and Ferro, V. O. (2006) 'Análise de própolis', *Food Science and Technology*, 26(1), 171-178. doi: 10.1590/S0101-20612006000100028

18. Peixoto, M. *et al*. (2021) 'Antioxidant and antimicrobial activity of blends of propolis samples collected in different years', *LWT*, 145, 111311. DOI: 10.1016/j.lwt.2021.111311

19. Bastos, E. M. A. F. *et al*. (2008) '*In vitro* study of the antimicrobial activity of Brazilian propolis against *Paenibacillus larvae*, *Journal of Invertebrate Pathology*, 97(3), 273-281. Doi: 10.1016/j.jip.2007.10.007

20. Coelho, J. *et al*. (2017) 'Phenolic composition and antioxidant activity assessment of southeastern and south Brazilian propolis', *Journal of Apicultural Research*, 56(1), 21-31. DOI: 10.1080/00218839.2016.1277602

21. Kumazawa, S. *et al*. (2013) 'Chemical and functional characterization of propolis collected from East Andalusia (Southern Spain)', *Phytochemical analysis*, 24(6), 608-615. DOI: 10.1002/pca.2439

22. Da Costa, L. A. M. A. *et al* (2016) 'Teor de fenólicos e atividade antioxidante de própolis em áreas de floresta e savana de Roraima', *RCT-Revista de Ciência e Tecnologia*, 2(3). doi: 10.18227/rct.v2i3.3869

23. Özkök, A. *et al*. (2021) 'Determination of antioxidant activity and phenolic compounds for basic standardization of Turkish propolis', *Applied Biological Chemistry*, 64(1), 1-10. DOI: 10.1186/s13765-021-00608-3

24. Mouhoubi-Tafinine, Z., Ouchemoukh, S., and Tamendjari, A. (2016) 'Antioxidant activity of some algerian honey and propolis', *Industrial Crops and Products*, 88, 85- 90. DOI: 10.1016/j.indcrop.2016.02.033

25. De Lima, V. H. M. *et al*. (2019a) 'Biological properties of volatile oil from Brazilian brown propolis', *Revista Brasileira de Farmacognosia*, 29(6), 807-810. DOI: 10.1016/j.bjp.2019.07.004

26. Calegari, M. A. (2018) 'Espectroscopia na região do infravermelho próximo (NIR) e calibração multivariada: desenvolvimento de modelos PLS para a determinação da atividade antioxidante em amostras de própolis', Masters dissertation, Federal Technological University of Paraná, Pato Branco. Available in: http://repositorio.utfpr.edu.br/jspui/handle/1/3199

27. Tiveron, A. P. *et al*. (2016) 'Chemical characterization and antioxidant, antimicrobial, and anti-inflammatory activities of South Brazilian organic propolis', *PLoS One*, 11(11). DOI: 10.1371/journal.pone.0165588

28. Kunrath, C. A.; Savoldi, D. C. (2014) 'Própolis como antioxidante em produtos cárneos: aplicação e avaliação em salame tipo italiano', Course conclusion monograph, Federal Technological University of Paraná. Available in: http://repositorio.roca.utfpr.edu.br/jspui/bitstream/1/3452/1/FB_COALM_2014_2_01.pdf.

29. Cavalaro, R. I. (2017) 'Atividade antioxidante de extratos de própolis verde em sistemas lipídicos emulsionados', Masters dissertation, University of São Paulo, Piracicabana. Available in:

https://www.researchgate.net/profile/Renata-Cavalaro/publication/346492928_Antioxidant_activity_of_green_propolis_extracts_in_systems_emulsified_lipids/links/5fc4fe57458515b7978ac762/Antioxidant-activity-of-green-propolis-extracts-in-systems-emulsified-lipids.pdf

30. De Francisco, L. *et al*. (2018) 'Evaluation of radical scavenging activity, intestinal cell viability and antifungal activity of Brazilian propolis by-product', *Food Research International*, 105, 537-547. DOI: 10.1016/j.foodres.2017.11.046

31. Ramón-Sierra, J. *et al*. (2019) 'Partial characterization of ethanolic extract of Melipona beecheii propolis and *in vitro* evaluation of its antifungal activity, *Revista Brasileira de Farmacognosia*, 29(3), 319-324. Doi: 10.1016/j.bjp.2019.04.002

32. Boonsai, P., Phuwapraisirisan, P., and Chanchao, C. (2014) 'Antibacterial activity of a cardanol from Thai *Apis mellifera* propolis', *International journal of medical sciences*, 11(4), 327. Doi: 10.7150/ijms.7373

33. Moher, David, *et al*. (2009) 'Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement', *PLoS medicine,* 6.7. DOI: 10.1371/journal.pmed.1000097

34. Castro, C. *et al*. (2014) 'Identification of phenolic compounds by HPLC-ESI-MS/MS and antioxidant activity from Chilean propolis', *Food Research International*, 64, 873-879. DOI: 10.1016/j.foodres.2014.08.050

35. Lacerda, R. C. C. (2012) 'Avaliação da composição química e atividade antioxidante da própolis orgânica de *Apis mellifera* visando à preservação ambiental do ecossistema envolvido', Master thesis, University of São Paulo, Piracicaba. Available in: https://www.teses.usp.br/teses/disponiveis/11/11141/tde-20122012-105318/en.php

36. Andrade, J. K. S. *et al*. (2018) 'Development and characterization of microencapsules containing spray-dried powder obtained from Brazilian brown, green and red propolis', *Food research international, Elsevier,* 109, 278-287. DOI: 10.1016/j.foodres.2018.04.048

37. Salgueiro, F. B. (2016) 'Caracterização da própolis verde brasileira: substâncias fenólicas, atividade biológica e análise quimiométrica', Doctoral thesis Federal Rural University of Rio de Janeiro, Seropédica. Available in: https://tede.ufrrj.br/handle/jspui/1469

38. Habermann, E. *et al*. (2016) 'Antioxidant activity and phenol content of extracts of bark, stems, and young and mature leaves from *Blepharocalyx salicifolius* (Kunth) O. Berg', *Brazilian Journal of Biology*, 76, 898-904. Doi: 10.1590/1519-6984.03815

39. Labyad, N. *et al*. (2016) 'Phytochemical antioxidant and antimicrobial study of Libyan propolis ethanolic extract', *International Journal of Herbal Medicine*, 4, 1-4.

40. Jansen, C. (2015*) '*Própolis: fitoquímicos e atividade antioxidante, antibacteriana e citotóxica', Master thesis, Federal University of Pelotas, Pelotas. Available in: http://guaiaca.ufpel.edu.br/handle/prefix/3881

41. Sanpa, S. *et al*. (2017) 'Chemical profiles and antimicrobial activities of Thai propolis collected from *Apis mellifera*', *Chiang Mai Journal of Science*, 44, 438-448.

42. Al Naggar, Y. *et al*. (2016) *'*Chemical characterization and antioxidant properties of Canadian propolis*'*, Journal of Apicultural Research. Taylor & Francis, 55(4), 305–314. DOI: 10.1080/00218839.2016.1233700

43. Andrade, J. K. S. *et al*. (2017) 'Evaluation of bioactive compounds potential and antioxidant activity of brown, green and red propolis from Brazilian northeast region', *Food Research International, Elsevier*, 101, 129-138. DOI: 10.1016/j.foodres.2017.08.066

44. Aranguena Salazar, E. (2019) 'Evaluación de la capacidad antioxidante y compuestos bioactivos del propóleo producido por abejas (*Apis mellifera*) recolectado por los métodos de raspado y rejilla de plástico en las comunidades campesinas de Huinchos Pataccocha y Chiara–provincia Andahuaylas', Professional Title Thesis of Agroindustrial Engineer, National University José María Arguedas, Andahuaylas.

45. Araújo, A. B. C. *et al*. (2020) 'Prospecção Química e Avaliação da Atividade Biológica da Própolis de Salinópolis, Pará', *Revista Virtual de Química*,12(2), 492-499. doi: 10.21577/1984-6835.20200039

46. Arruda, R. E. D. S. (2019) 'Efeito da sazonalidade na composição química e atividades antimicrobiana, antioxidante e tripanossomicida de extratos brutos de própolis vermelha de Alagoas', Masters dissertation. Federal University of Alagoas, Maceió. Available in: http://www.repositorio.ufal.br/bitstream/riufal/5565/1/Efeito%20da%20sazonalidade%20na%20composi

%C3%A7%C3%A3o%20qu%C3%ADmica%20e%20atividades%20antimicrobiana%2C%20antioxidante%20e%20tripanossomicida%20de%20extratos%20brutos%20de%20pr%C3%B3polis%20vermelha%20de%20Alagoas.pdf

47. Arslan, M. *et al*. (2021) 'Chemical and biological characteristics of propolis from *Apis mellifera caucasica* from the Ardahan and Erzurum provinces of Turkey: a comparative study, *Arhiv za higijenu rada i toksikologiju*, 72(1), 53-69.

48. Augusto-Obara, T. R. *et al*. (2019) 'Benefits of superfine grinding method on antioxidant and antifungal characteristic of Brazilian green propolis extract', *Scientia Agricola*, 76(5), 398-404. DOI: 10.1590/1678-992X-2018-0056

49. Bakkaloglu, Z., Arici, M., and Karasu, S. (2021) 'Optimization of ultrasound-assisted extraction of Turkish propolis and characterization of phenolic profile, antioxidant and antimicrobial activity, *Food Science and Technology*, 41(3). Doi: 10.1590/fst.14520

50. Béji-Srairi, R. *et al*. (2020) 'Ethanolic extract of Tunisian propolis: chemical composition, antioxidant, antimicrobial and antiproliferative properties, *Journal of Apicultural Research*, 59(5), 917-927. Doi: 10.1080/00218839.2020.1732572

51. Bhargava, H. R. *et al*. (2014) 'Antioxidative Analysis and Antagonistic Activity of *Apis* and *Trigona* Propolis Collected from Different Geo Locations', *Iranian (Iranica) Journal of Energy & Environment*, 5(4).

52. Bhuyan, D. J. *et al*. (2021) 'Broad-spectrum pharmacological activity of Australian propolis and metabolomic-driven identification of marker metabolites of propolis samples from three continents', *Food & Function*, 12(6), 2498-2519. DOI 10.1039/D1FO00127B

53. Boufadi, Y. M. *et al.* (2014) 'Characterization and antioxidant properties of six Algerian propolis extracts: ethyl acetate extracts inhibit myeloperoxidase activity, *International journal of molecular sciences*, 5(2), 2327-2345. DOI: 10.3390/ijms15022327

54. Cabral, I. S. R. *et al*. (2012) 'The correlation between the phenolic composition and biological activities of two varieties of Brazilian propolis (G6 and G12)', *Brazilian Journal of Pharmaceutical Sciences*, 48(3), 557-564. DOI: 10.1590/S1984-82502012000300023

55. Cavalaro, R. I., Fabricio, L. F. F., and Vieira, T. M. F. D. S. (2020) 'Ultrasound-Assisted Extraction of Antioxidants from *Baccharis dracunculifolia* and Green Propolis', *Processes*, 8(12), 1530. DOI: 10.3390/pr8121530

56. Cécere, B. G. O. *et al*. (2021) 'Addition of propolis to milk improves lactating lamb's growth: effect on antimicrobial, antioxidant and immune responses in animals', *Small Ruminant Research*, 194, 106265. DOI: 10.1016/j.smallrumres.2020.106265

57. Ceylan, Ö., and Halime, A. L. I. Ç. (2020) 'Antibiofilm, Antioxidant and Quorum Quenching Activities of Propolis Samples from Southwest Anatolia', *Bee Studies*, 12(2), 44-50. Doi: 10.51458/BSTD.2021.9

58. Coelho, J. P. M. (2013) 'Identificação e quantificação de compostos fenólicos em própolis da região sul do Brasil. Avaliação da atividade antioxidante por técnicas espectroscópicas e eletroquímicas', Masters dissertation, University of Salamanca, Bragança. Available in: https://bibliotecadigital.ipb.pt/handle/10198/10296

59. Correa, W. R. *et al*. (2016) 'ESI-MS fingerprinting of residues of green propolis, and evaluation of their antioxidant and antimicrobial activities, *Journal of Apicultural Research*, 55(1), 1-7. DOI: 10.1080/00218839.2016.1196014

60. Cottica, S. M. *et al*. (2011) 'Antioxidant activity and composition of propolis obtained by different methods of extraction', *Journal of the Brazilian Chemical Society*, 22(5), 929-935. DOI: 10.1590/S0103-50532011000500016

61. Cruz, M. A. O. (2011) 'Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis', Masters dissertation, University of Minho. Available in: https://repositorium.sdum.uminho.pt/handle/1822/18153

62. Da Cruz Almeida, E. T. *et al*. (2017) 'Chemical and microbiological characterization of tinctures and microcapsules loaded with Brazilian red propolis extract', *Journal of pharmaceutical analysis*, 7(5), 280-287. DOI: 10.1016/j.jpha.2017.03.004

63. da Graça Miguel, M. *et al*. (2014) 'Antioxidant, anti-inflammatory, and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco', *Food Science and Biotechnology*, 23(1), 313-322. DOI: 10.1007/s10068-014-0044-1

64. Dărăban, A. *et al*. (2019) 'The evaluation of antioxidant capaciti of propolis originating from Western Romania', *FARMACIA,* 67(1). doi: 10.31925/farmacia.2019.1.15

65. Da Silva, F. C. *et al*. (2013) 'Assessment of production efficiency, physicochemical properties and storage stability of spray-dried propolis, a natural food additive, using gum Arabic and OSA starch-based carrier systems', *Food and Bioproducts Processing*, 91(1), 28-36. DOI: 10.1016/j.fbp.2012.08.006

66. Da Silva, C. *et al*. (2018) 'Determination of total phenolic compounds and antioxidant activity of ethanolic extracts of propolis using ATR–FT-IR spectroscopy and chemometrics', *Food Analytical Methods*, 11(7), 2013-2021, 2018. DOI: 10.1007/s12161-018-1161-x

67. De Almeida, C. P. *et al*. (2017) 'Obtenção e caracterização de microencapsulados de caseinatos carregados com extrato de própolis vermelha', Masters dissertation, Federal University of Federal Alagoas, Maceió. Available in: http://www.repositorio.ufal.br/bitstream/riufal/1961/1/Obten%C3%A7%C3%A3o%20e%20caracteriza%C3%A7%C3%A3o%20de%20microencapsulados%20de%20caseinatos%20carregados%20com%20extrato%20de%20pr%C3%B3polis%20vermelha.pdf

68. De Lima, D. R. F. *et al*. (2019b) 'Avaliação das propriedades e potencialidades da própolis verde e sua fonte botânica *Baccharis dracunculifolia*', *Revista Tecnologia e Tendências*, 10(2), 93-110. doi: 10.25112/rtt.v10i2.2078

69. De Mendonça, I. C. G. (2014*)* 'Própolis vermelha alagoana: triagem fotoquímica, atividade antioxidante e citotoxidade em células de linhagens tumorais', Masters dissertation, CESMAC University Center, Maceió. Available in: https://ri.cesmac.edu.br/handle/tede/737

70. De Oliveira, S. C., Andolfatto, S. (2014) 'Otimização do processo de extração de compostos bioativos da própolis produzida por abelhas geneticamente modificadas', Course conclusion monograph, Federal Technological University of Paraná, Pato Branco. Available in: http://repositorio.utfpr.edu.br/jspui/bitstream/1/15288/2/PB_COQUI_2013_2_16.pdf

71. De Souza, E. C. A. *et al*. (2018) 'Chemical compositions and antioxidant and antimicrobial activities of propolis produced by *Frieseomelitta longipes* and *Apis mellifera* bees', *Química Nova*, 41(5), 485-491. DOI: 10.21577/0100-4042.20170208

72. Devequi-Nunes, D. *et al*. (2018) 'Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction', *PLoS One*, 13(12). DOI: 10.1371/journal.pone.0207676

73. Ding, Q. *et al*. (2021) 'Chinese Propolis: Ultrasound-assisted enhanced ethanolic extraction, volatile components analysis, antioxidant and antibacterial activity comparison', *Food Science & Nutrition*, 9(1), 313-330. DOI: 10.1002/fsn3.1997

74. Do Nascimento, T. G. *et al*. (2016) 'Polymeric Nanoparticles of Brazilian red propolis extract: preparation, characterization, antioxidant and leishmanicidal activity, *Nanoscale research letters*, 11(1), 1-16. DOI: 10.1186/s11671-016-1517-3

75. Do Nascimento, T. G. *et al*. (2019) 'Modified release microcapsules loaded with red propolis extract obtained by spray-dryer technique', *Journal of Thermal Analysis and Calorimetry*, 138(5), 3559-3569. DOI: 10.1007/s10973-019-08287-5

76. Duca, A. *et al*. (2019) 'Identification of resveratrol as bioactive compound of propolis from western Romania and characterization of phenolic profile and antioxidant activity of ethanolic extracts', *Molecules*, 24(18), 3368. DOI: 10.3390/molecules24183368

77. El Meniiy, N. *et al*. (2021) 'Influence of Geographic Origin and Plant Source on Physicochemical Properties, Mineral Content, and Antioxidant and Antibacterial Activities of Moroccan Propolis', *International Journal of Food Science*, 2021. DOI: 10.1155/2021/5570224

78. El Sohaimi, S. A., Masry, S. H. D (2014) 'Phenolic content, antioxidant and antimicrobial activities of Egyptian and Chinese propolis', *American-Eurasian Journal of Agricultural and Environmental Sciences*, 4, 1116-1124. DOI: 10.5829/idosi.aejaes.2014.14.10.8648

79. Ezzat, S. M. *et al*. (2019) 'Cytotoxicity, antioxidant, anti-inflammatory activity, and GC-MS analysis of Egyptian propolis', *Comparative Clinical Pathology*, 28(6), 1589-1598. DOI: 10.1007/s00580-019-02971-6

80. Fangio, M. F. *et al*. (2019) 'Chemical characterization and antimicrobial activity against Paenibacillus larvae of propolis from Buenos Aires province, Argentina', *Journal of Apicultural Research*, 58(4), 626-638. DOI: 10.1080/00218839.2019.1601318

81. Farias Azevedo, L. *et al*. (2018) 'Polymeric nanoparticle systems loaded with red propolis extract: a comparative study of the encapsulating systems, PCL-Pluronic versus Eudragit® E100-Pluronic', *Journal of Apicultural Research*, 57(2), 255-270. DOI: 10.1080/00218839.2017.1412878

82. Ferreira, J. M. (2019) 'Própolis e geoprópolis verde do semiárido do brasil: Caracterização química, origem botânica e atividade antioxidante', Doctoral thesis, Federal Rural University of the Semiarid Region, Mossoró. Available in: https://repositorio.ufersa.edu.br/handle/prefix/5182

83. Ferreira, J. M. *et al*. (2017) 'New propolis type from north-east Brazil: chemical composition, antioxidant activity, and botanical origin', *Journal of the Science of Food and Agriculture*, 97(11), 3552-3558. DOI: 10.1002/jsfa.8210

84. Galeotti, F. *et al*. (2017) 'Selective treatment to reduce contamination of propolis by polycyclic aromatic hydrocarbons (PAHs) still preserving its active polyphenol component and antioxidant activity, *Natural product research*, 31(17), 1971-1980. DOI: 10.1080/14786419.2016.1269093

85. Guo, X. *et al*. (2011) 'Chemical compositions and antioxidant activities of water extracts of Chinese propolis', *Journal of agricultural and food chemistry*, 59(23), 12610-12616. DOI: 10.1021/jf202818p

86. Hames-Kocabas, E. E. *et al*. (2013) 'Volatile composition of anatolian propolis by headspace solid-phase microextraction (HS-SPME), antimicrobial activity against food contaminants and antioxidant activity*, Journal of Medicinal Plants Research*, 7(28), 2140-2149. DOI: 10.5897/JMPR2013.4470

87. Ikeda, N. Y. (2020) 'Evaluation of the residues from the ethanolic extraction of organic propolis as a source of biological compounds', Doctoral thesis, University of São Paulo, Piracicaba. Available in: https://www.teses.usp.br/teses/disponiveis/11/11141/tde-04062020-084904/en.php

88. Irigoiti, Y., Yamul, D. K., and Navarro, A. S. (2021) 'Co-crystallized sucrose with propolis extract as a food ingredient: Powder characterization and antioxidant stability', *LWT*, 143, 111164. DOI: 10.1016/j.lwt.2021.111164

89. Jiang, X. *et al*. (2020) 'Grouping, Spectrum–Effect Relationship and Antioxidant Compounds of Chinese Propolis from Different Regions Using Multivariate Analyses and Off-Line Anti-DPPH Assay', *Molecules*, 25(14), 3243. DOI: 10.3390/molecules25143243

90. Jug, M., Končić, M. Z., and Kosalec, I. (2014) 'Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures', *LWT-Food Science and Technology*, 57(2), 530-537. DOI: 10.1016/j.lwt.2014.02.006

91. Kumar, M. R. *et al*. (2011) 'Antimicrobial and DPPH free radical-scavenging activities of the ethanol extract of propolis collected from India', *Journal of Ecobiotechnology*, 3(1).

92. Kumul, R. K. *et al*. (2020) 'Propolis of *Apis mellifera* from Yucatán, México: Study of biological properties', *Ecosistemas y Recursos Agropecuarios*, 7(3). doi: 10.19136/era.a7n3.2604

93. Kunrath, C. A. *et al*. (2017) 'Application and evaluation of propolis, the natural antioxidant in Italian-type salami', *Brazilian Journal of Food Technology*, 20. DOI: 10.1590/1981-6723.3516

94. Kurek-Górecka, A. M. *et al*. (2012) 'Antioxidant activity of ethanolic fractions of Polish propolis', *Zeitschrift für Naturforschung* C, 67(11-12), 545-550. doi: 10.1515/znc-2012-11-1203

95. Luis-Villaroya, A. *et al*. (2015) 'Bioactive properties of a propolis-based dietary supplement and its use in combination with mild heat for apple juice preservation', *International journal of food microbiology*, 205, 90-97. DOI: 10.1016/j.ijfoodmicro.2015.03.020

96. Machado, B. A. S. *et al*. (2016) 'Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil', *PloS one*, 11(1). DOI: 10.1371/journal.pone.0145954

97. Marcussi, M. C., and Gutierrez-Gonçalves, M. E. J. (2013) 'Atividades antimicrobiana e antioxidante da própolis do estado do Ceará', *Revista Fitos*, 4(1), 81-86.

98. Masek, A. *et al*. (2019) 'Electrochemical and spectrophotometric characterization of the propolis

antioxidants properties', *International Journal of Electrochemistry Science*, 14(2), 1231-1247. DOI: 10.20964/2019.02.66

99. Mello, B. C. B. S., and Hubinger, M. D. (2012) 'Antioxidant activity and polyphenol contents in Brazilian green propolis extracts prepared with the use of ethanol and water as solvents in different pH values, *International Journal of Food Science & Technology*, 47(12), 2510-2518. DOI: 10.1111/j.1365-2621.2012.03129.x

100. Mendez-Pfeiffer, P. *et al*. (2020) 'Seasonality Modulates the Cellular Antioxidant Activity and Antiproliferative Effect of Sonoran Desert Propolis', *Antioxidants*, 9(12), 1294. DOI: 10.3390/antiox9121294

101. Miguel, M.G. *et al*. (2014) 'Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal', *Food Science and Technology*, 34(1), 16-23.

102. Mohdaly, A. A. A. *et al*. (2015) 'Phenolic extract from propolis and bee pollen: composition, antioxidant and antibacterial activities, *Journal of Food Biochemistry*, 39(5), 538- 547. DOI: 10.1111/jfbc.12160

103. Molnár, S. *et al*. (2017) 'Comparative studies on polyphenolic profile and antimicrobial activity of propolis samples selected from distinctive geographical areas of Hungary', *Food Science and Technology International*, 23(4), 349-357. DOI: 10.1177/1082013217697469

104. Moncayo Luján, M. D. R. *et al*. (2018) 'Antibacterial activity and phenolic content of propolis extracts obtained by different extraction methods', *Nova scientia*, 10(20), 397-412. DOI: 10.21640/ns.v10i20.1392

105. Naik, D. G., and Vaidya, H. S. (2011) 'Antioxidant properties of volatile oil of Indian propolis', *Journal of ApiProduct ApiMedical Science*, 3(2), 89-93. DOI: 10.3896/IBRA.4.03.2.04

106. Nina, N. *et al*. (2015) 'Antibacterial activity, antioxidant effect and chemical composition of propolis from the Región del Maule, Central Chile', *Molecules*, 20(10), 18144-18167. DOI: 10.3390/molecules201018144

107. Nina, N. *et al*. (2016) 'Chemical profiling and antioxidant activity of Bolivian propolis', *Journal of the science of food and agriculture*, 96(6), 2142-2153. DOI: 10.1002/jsfa.7330

108. Nori, M. P. *et al*. (2011) 'Microencapsulation of propolis extract by complex coacervation', *LWT Food Science and Technology*, 44(2), 429-435. DOI: 10.1016/j.lwt.2010.09.010

109. Oldoni, T. L. C. *et al*. (2015) 'Chemical characterization and optimization of the extraction process of bioactive compounds from propolis produced by selected bees *Apis mellifera*', *Journal of the Brazilian Chemical Society*, 26(10), 2054-2062. DOI: 10.5935/0103-5053.20150186

110. Ozdal, T. *et al*. (2018) 'Evaluation of Turkish propolis for its chemical composition, antioxidant capacity, anti-proliferative effect on several human breast cancer cell lines and proliferative effect on fibroblasts and mouse mesenchymal stem cell line', *Journal of Apicultural Research*, 57(5), 627-638. DOI: 10.1080/00218839.2018.1494888

111. Ozdal, T. *et al*. (2019) 'Investigation of antioxidant capacity, bioaccessibility and LC-MS/MS phenolic profile of Turkish propolis', *Food Research International*, 122, 528-536. DOI: 10.1016/j.foodres.2019.05.028

112. Pazin, W. M. *et al*. (2017) 'Antioxidant activities of three stingless bee propolis and green propolis types', *Journal of Apicultural Research*, 56(1), 40-49. DOI: 10.1080/00218839.2016.1263496

113. Permana, A. D. *et al*. (2020) 'Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and photoprotective compounds in propolis: An approach for enhanced both dissolution behavior in biorelevant media and skin retention profiles', *Journal of Photochemistry and Photobiology B: Biology*, 205, 111846. DOI: 10.1016/j.jphotobiol.2020.111846

114. Prasniewski, A. (2015) 'Desenvolvimento de modelos de calibração multivariada para determinação de compostos fenólicos e atividade antioxidante em extrato etanólico de própolis utilizando a técnica FT-NIR', Course conclusion monograph, Federal Technological University of Paraná, Pato Branco. Available in: http://repositorio.roca.utfpr.edu.br/jspui/handle/1/5679.

115. Quintino, R. L. *et al*. (2020) 'Brazilian Green Propolis: Chemical Composition of Essential Oil and Their *In vitro* Antioxidant, Antibacterial and Antiproliferative Activities', *Brazilian Archives of Biology and*

Technology, 63. DOI: 10.1590/1678-4324-2020190408

116. Ramnath, S., and Venkataramegowda, S. (2016) 'Antioxidant activity of Indian propolis— An *in vitro* evaluation', *International Journal of Pharmacology, Phytochemistry, and Ethnomedicine*, 5, 79-85. DOI: 10.18052/www.scipress.com/IJPPE.5.79

117. Righi, A. A. *et al*. (2011) 'Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities, *Journal of the Science of Food and Agriculture*, 91(13), 2363-2370. DOI: 10.1002/jsfa.4468

118. Ristivojević, P. *et al*. (2018) 'Profiling of Turkish propolis subtypes: Comparative evaluation of their phytochemical compositions, antioxidant and antimicrobial activities', *LWT*, 95, 367-379. DOI: 10.1016/j.lwt.2018.04.063

119. Ristivojević, P. et al. (2020) 'Phenolic composition and biological activities of geographically different type of propolis and black cottonwood resins against oral streptococci, vaginal microbiota and phytopathogenic Fusarium species', Journal of applied microbiology, 129(2), 296-310. DOI: 10.1111/jam.14633

120. Rivera-Yañez, N. *et al*. (2018) 'Hypoglycaemic and antioxidant effects of propolis of Chihuahua in a model of experimental diabetes', *Evidence-Based Complementary and Alternative Medicine*, 2018. DOI: 10.1155/2018/4360356

121. Rocha, B. A. *et al*. (2013) 'Evaluation of a propolis water extract using a reliable RP-HPLC methodology and *in vitro* and efficacy and safety characterization', *Evidence-Based Complementary and Alternative Medicine*, 2013. DOI: 10.1155/2013/670451

122. Rodríguez, Y. *et al*. (2012) 'Physicochemical characterization and evaluation of antioxidant activity of propolis collected in the atlántic department, Colombia', *Revista UDCA Actualidad & Divulgación Científica*, 15(2), 303-311.

123. Rodríguez Pérez, B. *et al*. (2020) Chemical composition, antioxidant properties and antimicrobial activity of Mexican propolis', *Acta universitaria*, 30. DOI: 10.15174/au.2020.2435.

124. Salgueiro, F. B. and Castro, R. N. (2016) 'Comparação entre a composição química e capacidade antioxidante de diferentes extratos de própolis verde', *Química Nova*, 39(10), 1192-1199. DOI: 10.21577/0100-4042.20160136

125. Sánchez, R. D. V. *et al*. (2020) 'Effect of physicochemical properties and phenolic compounds of bifloral propolis on antioxidant and antimicrobial capacity, *Nova scientia*, 12(24). DOI: 10.21640/ns.v12i24.2134

126. Saral, Ö. *et al*. (2019) 'Evaluation of antioxidant activity of bee products of different bee races in Turkey', *Turkish Journal of Veterinary and Animal Sciences*, 43(4), 441-447. DOI: 10.3906/vet-1901-3

127. Schmidt, E. M. *et al*. (2014a) 'A comparison between characterization and biological properties of Brazilian fresh and aged propolis', *BioMed research international*, 2014. DOI: 10.1155/2014/257617

128. Schmidt, E. M. *et al*. (2014b) 'Effect of extraction solvent on antiradical activity of the obtained propolis extracts', *Journal of Apicultural Research*, 53(1), 91-100. DOI: 10.3896/IBRA.1.53.1.09

129. Shahbaz, M. *et al*. (2021) 'Chemical profiling, HPLC characterization and *in-vitro* antioxidant potential of Pakistani propolis collected from peripheral region of Faisalabad', *Cellular and Molecular Biology*, 67(1), 40-44. DOI: 10.14715/cmb/2021.67.1.6

130. Shehata, M. G. *et al*. (2020) 'Chemical analysis, antioxidant, cytotoxic and antimicrobial properties of propolis from different geographic regions', *Annals of Agricultural Sciences*, 65(2), 209-2017. DOI: 10.1016/j.aoas.2020.12.001

131. Shubharani, R., Mahesh, M., and Yogananda Murti, V. (2019) 'Biosynthesis and characterization, antioxidant and antimicrobial activities of selenium nanoparticles from ethanol extract of Bee Propolis', *Journal of Nanomedicine and Nanotechnology*, 10(1). DOI: 10.4172/2157-7439.1000522

132. Silva, F. R. G. *et al*. (2018) 'Phytochemical screening and *in vitro* antibacterial, antifungal, antioxidant and antitumor activities of the red propolis Alagoas', *Brazilian Journal of Biology*, 79(3), 452-459. DOI: 10.1590/1519-6984.182959

133. Sime, D. *et al*. (2015) 'Total phenols and antioxidant activities of natural honeys and propolis collected from different geographical regions of Ethiopia', *Bulletin of the Chemical Society of Ethiopia*, 29(2), 163-172. DOI: 10.4314/bcse.v29i2.1

134. Sousa, J. P. L. M. *et al*. (2019) 'Estudo químico e potencial antimicrobiano da própolis brasileira produzida por diferentes espécies de abelhas', *Revista Virtual de Química*, 11(5), 1480-1497. DOI: 10.21577/1984-6835.20190103

135. Sulaiman, G. M. *et al*. (2011) 'Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials', *Food and Chemical Toxicology*, 49(9), 2415-2421. DOI: 10.1016/j.fct.2011.06.060

136. Sun, C. *et al*. (2015) 'Effect of ethanol/water solvents on phenolic profiles and antioxidant properties of Beijing propolis extracts', *Evidence-Based Complementary and Alternative Medicine*, 2015. DOI: 10.1155/2015/595393

137. Svečnjak, L. *et al*. (2020) 'Mediterranean Propolis from the Adriatic Sea Islands as a Source of Natural Antioxidants: Comprehensive Chemical Biodiversity Determined by GC-MS, FTIR-ATR, UHPLC-DAD-QqTOF-MS, DPPH and FRAP Assay', *Antioxidants*, 9(4), 337. DOI: 10.3390/antiox9040337

138. Tiveron, A. P. (2015) 'Caracterização e identificação de compostos com atividade antioxidante de própolis orgânica brasileira', Doctoral thesis, University of São Paulo, Piracicaba. Available in: https://www.teses.usp.br/teses/disponiveis/64/64135/tde-04072016-151703/en.php

139. Toreti, V C. (2011) 'Estudo da influência da sazonalidade sobre algumas propriedades físico-químicas e biológicas da própolis de dois apiários do estado de São Paulo', Masters thesis, Estadual University of Campinas, Campinas. Available in: http://bdtd.ibict.br/vufind/Record/CAMP_d91baae3bdcdd30fdc2a34f6a8a1a9a6

140. Touzani, S. *et al*. (2018) 'Chemical analysis and antioxidant content of various propolis samples collected from different regions and their impact on antimicrobial activities', *Asian Pacific Journal of Tropical Medicine*, 11(7), 436. DOI: 10.4103/1995-7645.237188

141. Vargas-Sánchez, R. D. *et al*. (2019) 'Propolis extract as antioxidant to improve oxidative stability of fresh patties during refrigerated storage', *Foods*, 8(12), 614. DOI: 10.3390/foods8120614

142. Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., and Sánchez Escalante, A. (2020) 'Physicochemical and microbiological characterization, and evaluation of the antibacterial and antioxidant activity of propolis produced in two seasons and two areas of the eastern edge of the Sonoran Desert', *Biotecnia*, 22(3), 46-52. DOI: 10.18633/biotecnia.v22i3.1185

143. Venegas, Y. *et al*. (2016) 'A new near-infrared method for simultaneous determination of caffeic acid phenethyl ester and antioxidant activity of propolis samples', *Journal of Apicultural Research*, 55(1), 8-18. DOI: 10.1080/00218839.2016.1192374

144. Wali, A. F. *et al*. (2016) '*In vitro* antioxidant and antimicrobial activities of propolis from Kashmir Himalaya region', *Free radicals and antioxidants*, 6(1), 51-57. DOI: 10.5530/fra.2016.1.6

145. Wang, X. *et al*. (2016) 'Relationship between total phenolic contents and biological properties of propolis from 20 different regions in South Korea', *BMC complementary and alternative medicine*, 16(1), 1-12. DOI: 10.1186/s12906-016-1043-y

146. Wiwekowati, W. *et al*. (2017) 'Antioxidant activity of *Apis mellifera* sp. Propolis extract from Java (Indonesia)', *International Research Journal of Engineering*, *IT and Scientific Research*, 3(5), 19-24.

147. Xavier, J. D. A. *et al*. (2017) 'Polyphenol profile by UHPLC-MS/MS, anti-glycation, antioxidant and cytotoxic activities of several samples of propolis from the northeastern semi-arid region of Brazil', *Pharmaceutical biology*, 55(1), 1884-1893. DOI: 10.1080/13880209.2017.1340962

148. Yuan, M. *et al*. (2020) 'A comparative study between Chinese propolis and Brazilian green propolis: metabolite profile and bioactivity', *Food & function*, 11(3), 2368-2379. DOI: 10.1039/c9fo02051a

149. Yurteri, Ü. (2015) 'Investigation of bioactivity and chemical content of Turkish propolis from Ankara province', Master thesis, Middle East Technical University. Available in: https://open.metu.edu.tr/handle/11511/25377

150. Zeitoun, R. *et al*. (2019) 'Chemical composition, antioxidant and anti-inflammatory activity evaluation of the Lebanese propolis extract', *Current pharmaceutical biotechnology*, 20(1), 84-96. DOI: 10.2174/1389201020666190206201241

151. Zhang, J. *et al*. (2015) 'Comparisons of ethanol extracts of Chinese propolis (poplar type) and poplar gums based on the antioxidant activities and molecular mechanism', *Evidence-Based Complementary*

and Alternative Medicine, 2015. DOI: 10.1155/2015/307594

152. Zhang, J. *et al*. (2016) 'Antioxidant activities and molecular mechanisms of the ethanol extracts of *Baccharis* propolis and Eucalyptus propolis in RAW64. 7 cells', *Pharmaceutical Biology*, 54(10), 2220-2235. DOI: 10.3109/13880209.2016.1151444

153. Žižić, J. B. *et al*. (2013) 'Chemical composition, cytotoxic and antioxidative activities of ethanolic extracts of propolis on HCT-116 cell line', *Journal of the Science of Food and Agriculture*, 93(12), 3001-3009. DOI: 10.1002/jsfa.6132

154. De Morais, D. V. *et al*. (2021) 'Active antioxidant phenolics from Brazilian red propolis: an optimization study for their recovery and identification by LC-ESI-QTOF-MS/MS', *Antioxidants*, 10(2), 297. DOI: 10.3390/antiox10020297

155. Silva, V. *et al*. (2011) 'Antioxidant activity of Uruguayan propolis. *In vitro* and cellular assays', *Journal of Agricultural and Food Chemistry*, 59(12), 6430-6437. DOI: 10.1021/jf201032y

156. Vargas Tapia, E. (2018) 'Identificação da fonte botânica, caracterização química e avaliação das atividades biológicas das própolis coletadas no Peru', Doctoral thesis, Estadual University of Campinas, Campinas. Available in: https://1library.org/document/q7rnjkny-identificacao-botanica-caracterizacao-avaliacao-atividades-biologicas-propolis-coletadas.html

157. Abubaker, E. A., and Fageer, A. S. M. (2017) 'Antioxidant capacity and mineral content of some sudanese propolis', *International Journal of Science and Research*, 6(2), 1621-1626. DOI: 10.21275/ART2017940

158. Ertürk, Ö. *et al*. (2016) 'An *in vitro* study on antimicrobial and antioxidant activity of propolis from Rize Province of Turkey', *Mellifera*, 16(1), 4-18.

159. Gokduman, K. (2019) '*In vitro* investigation of anti-cancer activity of propolis on hepatocellular carcinoma cells', *Medicine*, 8(3), 557-62. DOI: 1 0.5455/medscience.2019.08.9013

160. Kiziltas, H.; Erkan, C. (2020) 'The effects of different beehives on propolis production and quality', *Food Science and Technology*, 41(4). DOI: 10.1590/fst.20120

161. Özyürek, M. *et al*. (2011) 'A comprehensive review of CUPRAC methodology', *Analytical methods*, 3(11), 2439-2453.

162. Walker, R. B., and Everette, J. D. (2009) 'Comparative reaction rates of various antioxidants with ABTS radical cation', *Journal of agricultural and food chemistry*, 57(4),1156- 1161. DOI: 10.1021/jf8026765

163. Nenadis, N. *et al*. (2004) 'Estimation of scavenging activity of phenolic compounds using the ABTS•+ assay', *Journal of agricultural and food chemistry*, 52(15), 4669-4674. DOI: 10.1021/jf0400056

164. Salas, A. L. *et al*. (2016) 'Biological activities of polyphenols-enriched propolis from Argentina arid regions', *Phytomedicine*, 23(1), 27-31. DOI: 10.1016/j.phymed.2015.11.007

165. Boulechfar, S. *et al*. (2019) 'Investigation of Antioxidant and Anticholinesterase Potential of Essential Oil and Methanolic Extract of Propolis from Mila Region', *Journal of Biologically Active Products from Nature*, 9(6), 434-444. DOI: 10.1080/22311866.2019.1703816

166. Da Cunha, G. F. (2017*)* 'Biofilmes à Base de Amido Incorporados com Extrato Etanólico de Própolis', Masters dissertation, Goiano Federal Institute of Education, Science and Technology, Rio Verde. Available in: https://repositorio.ifgoiano.edu.br/handle/prefix/40

167. Del Río Del Rosal, E. *et al*. (2017) 'Estudio de la actividad antioxidante y antitumoral del propóleo', *Ars Pharmaceutica*, 58(2), 75-81. DOI: 10.4321/s2340-98942017000200005

168. Gargouri, W. *et al*. (2019) 'Evaluation of bioactive compounds and biological activities of Tunisian propolis', *Lwt*, 111, 328-336. DOI: 10.1016/j.lwt.2019.05.044

169. Osés, S. M. *et al*. (2016) 'Bioactive properties of honey with propolis', *Food chemistry*, 196, 1215-1223. DOI: 10.1016/j.foodchem.2015.10.050

170. Osés, S. M. *et al*. (2020) 'Phenolic profile, antioxidant capacities and enzymatic inhibitory activities of propolis from different geographical areas: Needs for analytical harmonization', *Antioxidants*, 9(1), 75. DOI: 10.3390/antiox9010075

171. Salas, A. *et al*. (2018) 'Development of a bioproduct for medicinal use with extracts of Zuccagnia type propolis', *Natural Product Communications*, 13(2). DOI: 10.1177/1934578X1801300214.

172. Salas, A. L. *et al*. (2020) 'Zuccagnia-type Propolis from Argentina: A potential functional ingredient in food to pathologies associated to metabolic syndrome and oxidative stress', *Journal of Food Science*, 85(8), 2578-2588. DOI: 10.1111/1750-3841.15323

173. Yonar, S. M. *et al*. (2017) 'Effects of dietary propolis on the number and size of pleopadal egg, oxidative stress and antioxidant status of freshwater crayfish (*Astacus leptodactylus Eschscholtz*)', *Animal reproduction science*, 184, 149-159. DOI: 10.1016/j.anireprosci.2017.07.010

174. Ferreira, A. L. A., and Matsubara, L. S. (1997) 'Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo', *Revista da associação médica brasileira*, 43(1), 61-68. DOI: 10.1590/S0104-42301997000100014

175. Isla, M. I. *et al*. (2009) 'Effect of seasonal variations and collection form on antioxidant activity of propolis from San Juan, Argentina', *Journal of medicinal food*, 12(6), 1334-1342. DOI: 10.1089/jmf.2008.0286

176. Silva, F. A. M., Borges, M. F. M., and Ferreira, M. A. (1999) 'Métodos para avaliação do grau de oxidação lipídica e da capacidade antioxidante', *Química nova*, 22(1), 94-103. DOI: 10.1590/S0100-40421999000100016

177. Oliveira, G. L. D. S. (2015) 'Determinação da capacidade antioxidante de produtos naturais *in vitro* pelo método do DPPH• : estudo de revisão', *Revista Brasileira de Plantas Medicinais*, 17, 36-44, 2015. DOI: 10.1590/1983-084X/12_165

178. Graikou, K. *et al*. (2016) 'Characterization and biological evaluation of selected Mediterranean propolis samples. Is it a new type', *LWT-Food Science and Technology*, 65, 261-267. DOI: 10.1016/j.lwt.2015.08.025

179. Jeong, C. H. *et al*. (2012*) '*Phenolics of propolis and *in vitro* protective effects against oxidative stress-induced cytotoxicity', *Journal of Agriculture and Life Science*, 46(3), 87-95.

180. Sousa, R. M. F. *et al*. (2014) 'Atividade antioxidante de extratos de folhas de ora-pro-nóbis (*Pereskia aculeata* Mill.) usando métodos espectrofotométricos e voltamétricos *in vitro*', *Bioscience Journal*, 30(3).

181. Reis, N. S. *et al*. (2009) 'Métodos eletroquímicos usados para avaliação da atividade antioxidante de produtos naturais', *Latin American Journal of Pharmacy*, 28.

182. De Oliveira, T. I. F. (2015) 'Efeitos tóxicos de amostras de própolis português: potencial antioxidante e atividades biológicas de extratos e misturas', Masters dissertation, University of Minho. Available in: https://repositorium.sdum.uminho.pt/handle/1822/37479

183. Koury, J. C., and Donangelo, C. M. (2003) 'Zinco, estresse oxidativo e atividade física', *Revista de Nutrição*, 16, 433-441. DOI: 10.1590/S1415-52732003000400007

184. Yonar, M. E. *et al*. (2012) 'Protective role of propolis in chlorpyrifos-induced changes in the haematological parameters and the oxidative/antioxidative status of *Ciprinus carpio carpio'*, *Food and Chemical Toxicology*, 50(8), 2703-2708. DOI: 10.1016/j.fct.2012.05.032

185. Marques, R. A. C. (2015) 'Contributos para a elucidação do modo de ação de própolis português: o caso doprópolis do Pereiro', Master thesis, University of Minho. Available in: https://repositorium.sdum.uminho.pt/handle/1822/41145

186. Fadaka, A. O. *et al*. (2019) 'Significance of antioxidants in the treatment and prevention of neurodegenerative diseases', *The Journal of Phytopharmacology*, 8(2), 75-83.

187. El-Guendouz, S. *et al*. (2016) 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', *International Journal of Food Science & Technology*, 51(8), 1762-1773. DOI: 10.1111/ijfs.13133

188. Da Silveira, C. C. S. D. M. *et al*. (2016) 'Neurobehavioral and antioxidant effects of ethanolic extract of yellow propolis', *Oxidative medicine and cellular longevity*, 2016. DOI: 10.1155/2016/2906953

189. Valente, M. J. *et al*. (2011) 'Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth *in vitro'*, *Food and Chemical Toxicology*, 49(1), 86-92. DOI: 10.1016/j.fct.2010.10.001

190. Capucho, C. *et al*. (2012) 'Green Brazilian propolis effects on sperm count and epididymis morphology and oxidative stress', *Food and chemical toxicology*, 50(11), 3956-3962. DOI: 10.1016/j.fct.2012.08.027

191. Yonar, S. M. *et al*. (2014) 'Malathion-induced changes in the haematological profile, the immune response, and the oxidative/antioxidant status of *Cyprinus carpio carpio*: Protective role of propolis', *Ecotoxicology and environmental safety*, 102, 202-209. DOI: 10.1016/j.ecoenv.2014.01.007

192. Saleh, E. M. (2012) 'Antioxidant effect of aqueous extract of propolis on hepatotoxicity induced by octylphenol in male rats', *Acta Toxicológica Argentina*, 20(2), 68-81.

193. Tohamy, A. A. *et al*. (2014) 'Assessment of anti-mutagenic, anti-histopathologic and antioxidant capacities of Egyptian bee pollen and propolis extracts', *Cytotechnology*, 66(2), 283- 297. DOI: 10.1007/s10616-013-9568-0

194. Wiwekowati, W. *et al*. (2020) 'Indonesian Propolis Reduces Malondialdehyde Level and Increase Osteoblast Cell Number in Wistar Rats with Orthodontic Tooth Movement', *Open Access Macedonian Journal of Medical Sciences*, 8,100-104. DOI: 10.3889/oamjms.2020.3984

195. Karadal, F. *et al*. (2018) 'A study of antibacterial and antioxidant activities of bee products: Propolis, pollen and honey samples', *Ethiopian Journal of Health Development*, 32(2).

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Review Article

STABILITY OF FEVERFEW AND ITS ACTIVE PRINCIPLE PARTHENOLIDE: AN ELUSIVE ANTIMIGRAINE HERBAL MEDICINE

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ABSTRACT

Background

Feverfew is a traditional herbal remedy for the relief of arthritis, migraine, toothache, and menstrual difficulties. It is widely accepted that parthenolide, a sesquiterpene lactone, is its main active principle. However, the decrease of parthenolide in commercial preparations is a well-known process with no technical solution so far.

Aims

To review the evidence for the mechanism of the degradation of parthenolide and similar sesquiterpene lactones.

Methods

Systematic review.

Results and Conclusion

In conclusion, and without discarding any degradation of parthenolide into non-identifiable fragments, the fate of this compound in dry, powdered feverfew is to undergo a covalent binding to plant proteins resulting in a biologically inactive adduct - in accordance with the direct and indirect data found in the literature. This process seems to be virtually unstoppable, and temperature and light do not seem to be playing a significant role under normal storage conditions according to some authors. In the presence of a high level of humidity, parthenolide may undergo an acid-induced cyclisation giving rise to a guaianolide-type sesquiterpene lactone, a class of compound that is commonly found in Feverfew. Microbial degradations are not likely to play an important role if the formulation complies with Pharmacopoeial microbiological quality requirements. The experimental and clinical data in the literature do not report on any increase in the toxicity of stored feverfew.

Keywords

Sesquiterpene lactones, Chemical stability, Herbal medicines, Microbial degradation, Storage.

INTRODUCTION: FEVERFEW AND PARTHENOLIDE

Feverfew is a traditional herbal remedy for the relief of arthritis, migraine, toothache, and menstrual difficulties. These properties have solid pharmacological foundations (Wang & Li, 2015) and many feverfew-based medicines are regulated and sold in Europe under the Traditional Herbal Medicinal Products Directive. The plant is a perennial, strongly aromatic herb of the Compositae/Asteraceae family, and has been classified variously as *Tanacetum parthenium*, C*hrysanthemum parthenium*, *Leucanthemum parthenium*, or *Pyrethrum parthenium*, the former name being currently favoured. Of a range of sesquiterpene lactones of the germacrane (germacranolides) and guianane (guaianolides) groups characterized in the leaf material, the principal constituent and major active component is parthenolide (Figure 1). Parthenolide (CAS 20554-84-1) which can occur in up to 1%*w/w* in dried leaves, is a white crystalline solid with MW=248.3 and melting point of 112-115ºC. In comparison with other sesquiterpene lactones, parthenolide is remarkably soluble in water as well as in organic solvents (amphiphilic) and large quantities can be extracted into aqueous buffers (Heptinstall, 1998). The amount of parthenolide in feverfew depends on environmental conditions, feverfew chemovars, part of the plant and postharvest processing (Majdi et al.2013). Parthenolide content is higher in flowers, followed by leaves and stems but absent in roots (Majdi et al.2011). Other sesquiterpene lactones with an α-methylenebutyrolactone ring present in feverfew are canin, artecanin, tanaparthin-α- and -β peroxides, and seco-tanapartholide A and B (Figure 1) (Begley et al, 1989).

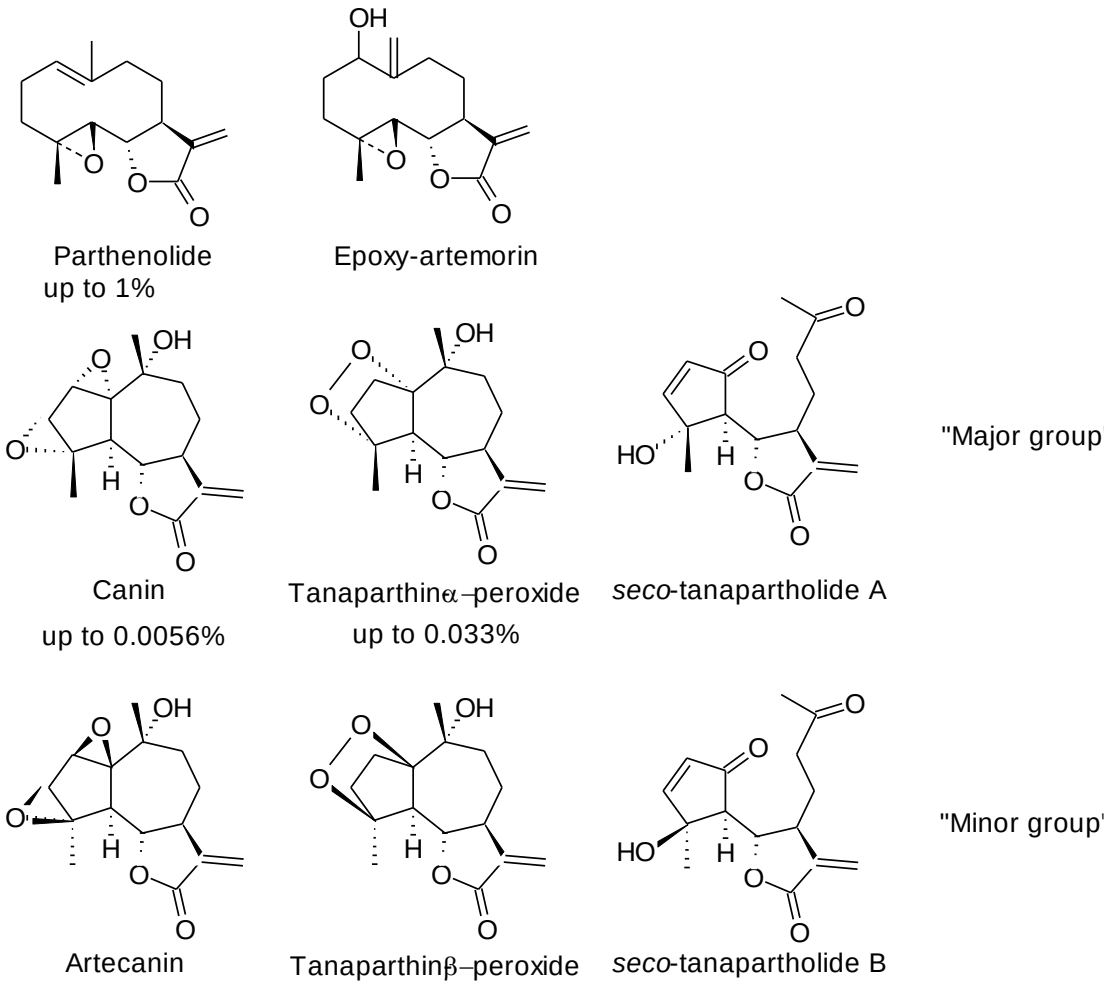

Figure 1. Main sesquiterpene lactones with α-methylenebutyrolactone group found in Feverfew.

THE EFFECT OF STORAGE OF FEVERFEW ON THE CONTENT OF PARTHENOLIDE.

The decrease of parthenolide in commercial preparations is a well-known process (Knight, 1995; Heptinstall and Awang, 1998) with no technical solution until now. The loss of parthenolide does not seem to be significantly affected by temperature or light exposure during storage (Fonseca et al, 2003; Tanko et al, 2003), so little can be done by controlling these parameters. Groenewegen et al (1992) reported fourteen years ago that there is little information on the stability of the sesquiterpene lactones during storage. The appearance of costunolide diepoxide when a chloroform solution of parthenolide was stored at room temperature after a few days, probably formed by aerial oxidation (Hoffman et al, 1977), and the suggestion that parthenolide can polymerize on prolonged storage were the only two references they could cite in their paper (Groenewegen et al, 1992). Since then, little work has been published and still today only indirect information is available. However, it is well known that the parthenolide content of dried leaf decreases on storage (Heptinstall et al, 1992).

Other herbal formulations containing sesquiterpene lactones face the same problems, the most important example coming from Arnica-containing formulations. Lactone-containing herbal remedies such as *Arnica spp.* preparations are frequently used in phytotherapy and sesquiterpene lactones are the main active compounds. When tinctures of *Arnica spp.* were stored at 4ºC, 25ºC and 30ºC, a decrease in the content of 11α, 13-dihydrohelenalin esters correlating with the temperature was observed. It was caused by addition of ethanol to the cyclopentenone moiety of these molecules (Schmidt et al, 2000). Bilia and co-workers (2006) studied the stability of lactones in different semisolid formulations under different conditions of storage and found great differences depending on the different excipients used. As a result, the cetomacrogol, polysorbate 60, and natrosol-based formulations were the least stable.

Several studies have been carried out to determine the effect of drying temperature and storage of feverfew on the concentration of parthenolide as its marker compound. Studies have examined the analysis of parthenolide using HPLC-UV after extraction from the feverfew leaves which had been dried, comminuted, and extracted. Drying the leaves at temperatures in the range of 30 to 60°C did not significantly affect the parthenolide concentration (Tanko et al, 2003). These authors also found that storage temperature in the range of -15 to 24°C did not significantly affect parthenolide concentration either. However, parthenolide concentration decreased as a function of time over 120 days of storage. In another study, Fonseca and co-workers (2003) reported that exposure to light (sunlight, or fluorescent light) in the drying process actually increases the content of parthenolide. Furthermore, light did not affect either dry feverfew powder or pure parthenolide during storage. Surprisingly, exposure of the stored material to heat (50-130ºC) during a short time (5 min) increases the extraction yield of parthenolide. In contrast these workers observed a drastic loss of parthenolide if the powder was dissolved in citrate buffers at various pH values (2.4 to 7.2).

Feverfew extract dissolved in different pH buffers to study the solution stability of parthenolide in feverfew. The degradation of parthenolide follows a first-order reaction model and is stable in the range of 5 to 7, becoming unstable when pH is less than 3 or more than 7. In solid feverfew extracts, moisture content and temperature are the most important parameters influencing the rate of parthenolide degradation which does not fit any obvious reaction model. Up to 40% loss in parthenolide were observed when extracts are stored at 50°C/31% RH and these losses go down to 18% to 32% after 6-month storage under 40°C/ 0% to 75% RH. When feverfew is mixed with excipients parthenolide levels remain constant at 5°C/31% RH for up to 6 months and exhibits good compatibility with common excipients under stress conditions up to 3-weeks (Jin et al, 2007).

Fonseca and co-workers (2007) studied the influence of pH, temperature and light on parthenolide content in powdered feverfew and parthenolide standard in citrate buffers at selected pH (2.4-7.2) were stored for 4 months. Parthenolide losses in solutions are greater at pH<5, and lower at pH=7.2. Parthenolide losses in dry samples were 30% percent after 320 days of storage. Short term studies (hours) show stability of the compound even at high temperatures though. Similar results were reported by Marete et al. (2011, 2013) in feverfew model beverages. Parthenolide hydrolysis increases with acidity and neutral infusions (pH 6.0) favour its stability but favour oxidation of phenolic substances. The best conditions are refrigerated storage -as parthenolide degradation with thermal treatment follows pseudo-first order kinetics- of mildly acidic infusions (pH 4.6) for both colour retention and parthenolide content with a shelf-life of approximately 4 months.

Finally, studies with “degraded” feverfew extracts found that they were pharmacologically active in an *in vivo* murine model of anti-serotonergic activity up to a 10% of loss of parthenolide (Mittra et al, 2000). The same authors did not report any toxic effect on rats treated with extracts degraded with up to a 33% loss of parthenolide.

REACTIVITY OF PARTHENOLIDE, GERMACRANOLIDES AND RELATED SESQUITERPENE LACTONES.

Generalities

It is a characteristic of sesquiterpenes to undergo *in vitro* acid-catalysed cyclizations. Cyclizations play a central role in the biosynthesis of guaianolides and eudesmanolides from germacranolides in the normal plant cell metabolism under enzymatic control (Cane, 1999), but they can also be reproduced *in vitro* in the presence of acids (Sethi et al, 1984; Castaneda- Acosta et al, 1993). In addition, germacranolides and related sesquiterpene lactones are frequently endowed with α, β unsaturated carbonyl groups making these molecules especially prone to react with nucleophiles following a Michael-type addition. The α, β unsaturated carbonyl groups is usually in the form of an α-methylene-γ-lactone group, also called an α-methylenebutyrolactone, and cyclopentenones. Other functional groups that are usually present in sesquiterpene lactones are endocyclic double bonds and epoxides, which can both react with electrophiles and/or induce rearrangements in the presence of UV radiation. Finally, high temperatures and strong irradiation may cause Cope rearrangements.

It is worth pointing out that reactivity does not necessarily infer chemical instability. The α-methylenebutyrolactone group – responsible for many of the biological activities of STLs -and possibly for their disappearance from the plant material over time- is often wrongly regarded as an unstable chemical feature. However, it is postulated that the exo double bond stabilizes the 5- membered ring of these sesquiterpenes. In fact, the biosynthesis of these compounds seems to proceed in such a manner as to favour the formation or retention of an exo double bond in a 5- membered ring and to avoid the formation or retention of an exo double bond in a 6-membered ring. With a few exceptions this generalization is supported by available data on the stability of cyclic esters, lactones, hemiacetals, and imides, and even in furanose and pyranose structures in the sugars and sugar acids. This generalization is in accord with available thermochemical data demonstrating that an atom of oxygen in a ring system can result in the same type of conformational effects as a CH_2 group (Brown et al, 1954). The lactone ring also has a strong influence on the thermal stability of germacrenes (de Kraker et al, 2000).

In vitro acid-catalysed cyclizations

Many sesquiterpene lactones, particularly germacranolides, and therefore parthenolide, undergo facile *in vitro* acid-catalysed cyclizations (Figure 2). Their own biosynthesis is based on a series of cyclizations, starting with farnesyl diphosphate, by electrophilic attack on to an appropriate double bond. Standard reactions of carbocations rationally explain most of the common structural skeletons encountered (Dewick, 2002). The formation of guaianolides or eudesmanolides probably occurs by cyclization of (+)-costunolide after C_4-C_5 epoxidation. or C_1-C_{10} epoxidation, respectively (Figure 2) (Piet et al, 1995; de Kraker et al, 2002).

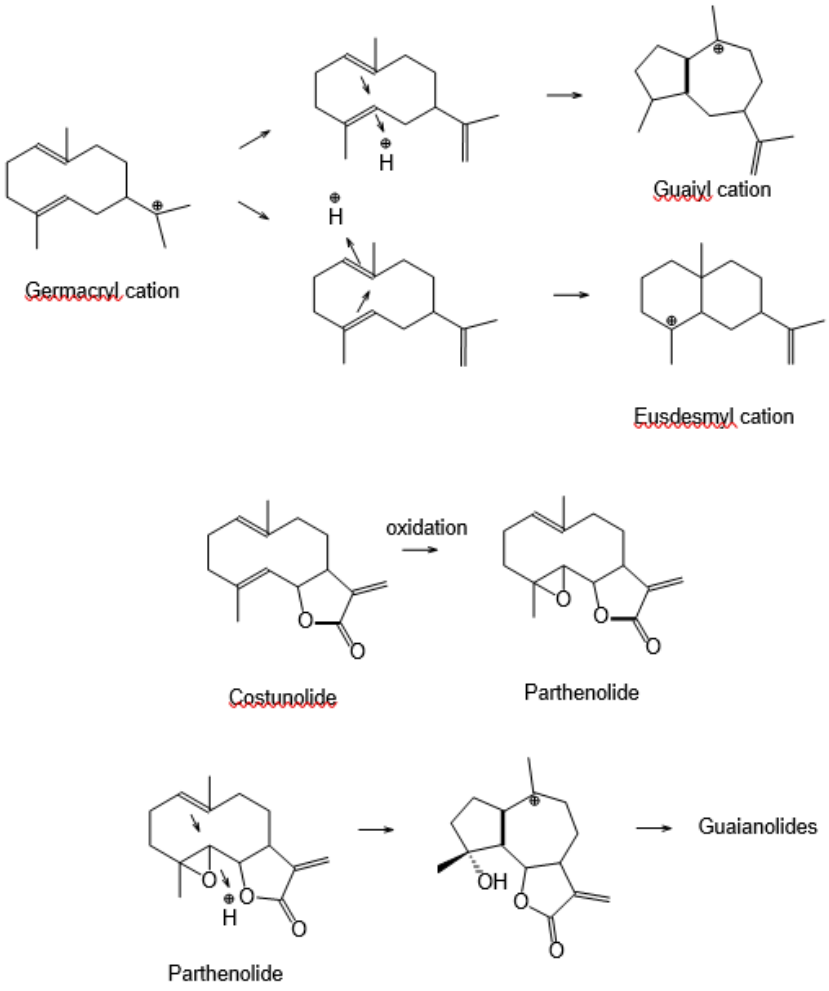

Figure 2. Mechanisms and intermediates of *in vivo* acid-catalysed cyclizations of germacranes and parthenolide.

Sethi and co-workers (1984) briefly reported the conversion of parthenolide into guaianolides after BF_3-catalyzed cyclization. A fuller description of these conversions was given by Castaneda-Acosta and co-workers (1993), by using the *in vitro* BF_3-mediated biomimetic approach in hexane. They explained the mechanisms as rearrangements involving carbocation intermediates giving rise to the major product micheliolide, and several minor products namely 10(14)-dehydro-5α-hydroxy-*trans*-guaianolide, 9,10-dehydro-5α-hydroxy-*trans*-guaianolide, the xanthanolide 2-desoxy-6-*epi*-parthemollin, 1,2-dehydro-4α-hydroxyguaianolide, 11,13-dehydrocompressanolide, and bicyclo[6.2.0]dec-10(14)-en-12,6-olide (Figure 3). All the identified products preserve the α-methylene-γ-lactone group.

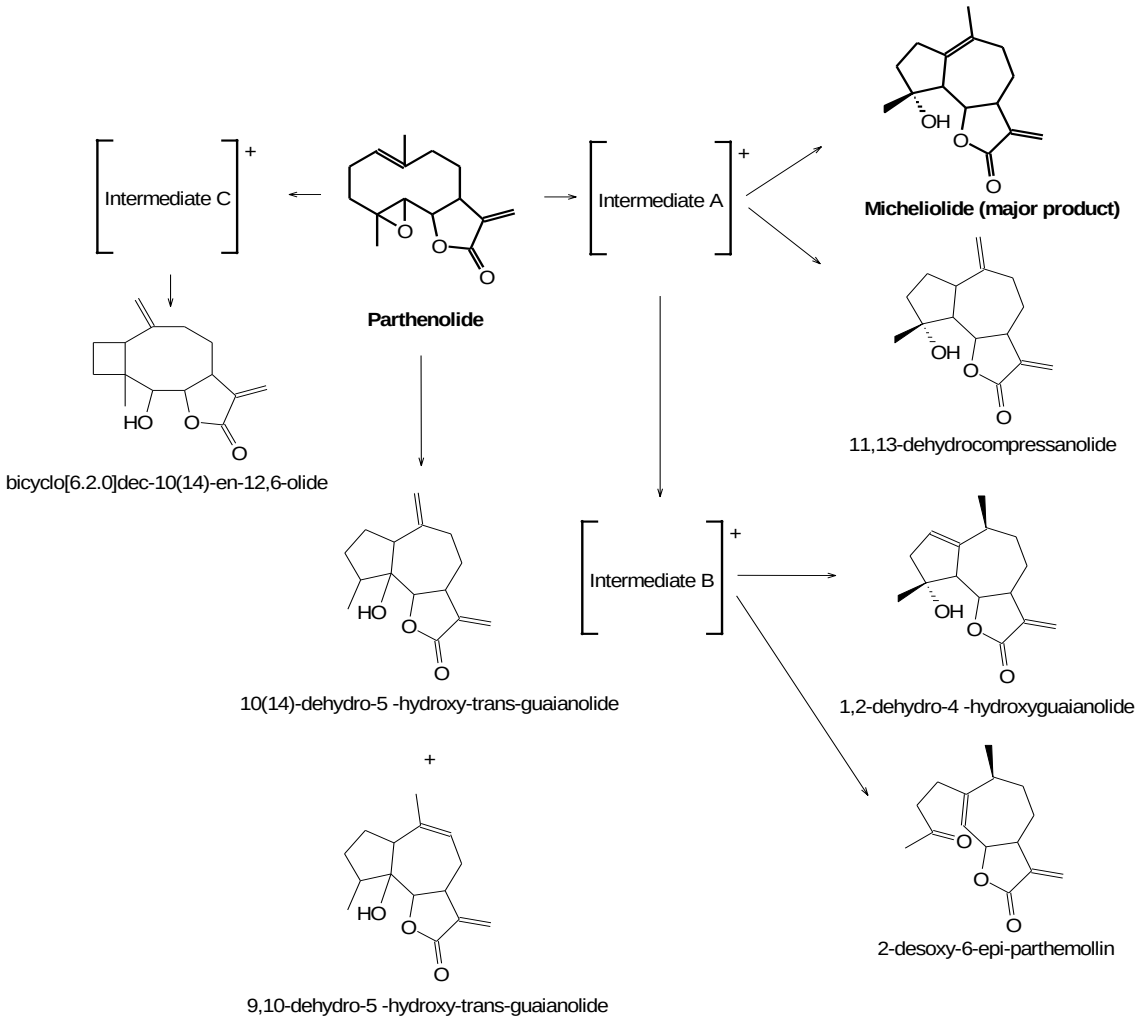

Figure 3. Products obtained after BF_3-initiated biomimetic cyclisations of parthenolide (As described by Castaneda-Acosta et al, 1993)

The cyclization of parthenolide in an aqueous acidic environment with R-OH acting as a nucleophile does not seem to be favoured but it may occur in the way depicted in Figure 4. If R=H, that is water is acting as the nucleophile, the resulting product is the guainolide partholide, that has been reported to be present in feverfew according to Johnson and co-workers (1982).

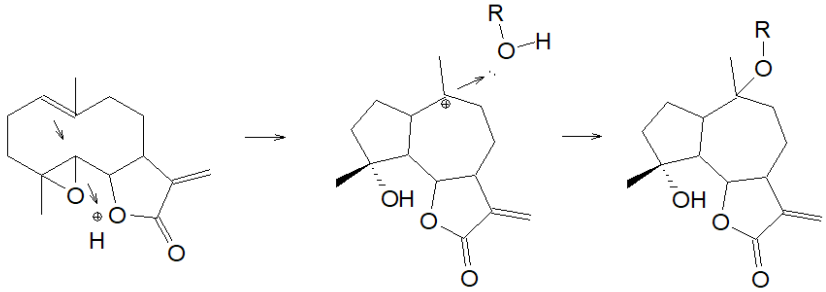

Figure 4. Cyclization of parthenolide in an aqueous acidic environment with R-OH acting as a nucleophile.

The possibility of a cyclization of parthenolide giving rise to a trans-decalin system, thus forming an eudesmanolide, (Figure 5) seems to be less likely as the carbocation cannot be stabilised on a tertiary carbon.

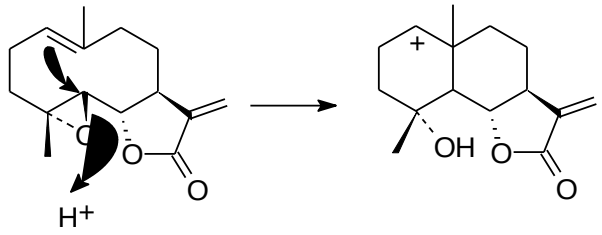

Figure 5. Cyclization of parthenolide giving rise to a trans-decalin system.

Nevertheless, such a type of transannular cyclization could be possible, and similar rearrangements have been reported to occur *in vitro with c*ostunolide, the analogue of parthenolide lacking the epoxide group. Costunolide can be smoothly converted into α- and β- cyclocostunolide by Amberlite cation exchange resin (Figure 6) (Roberts, 1972).

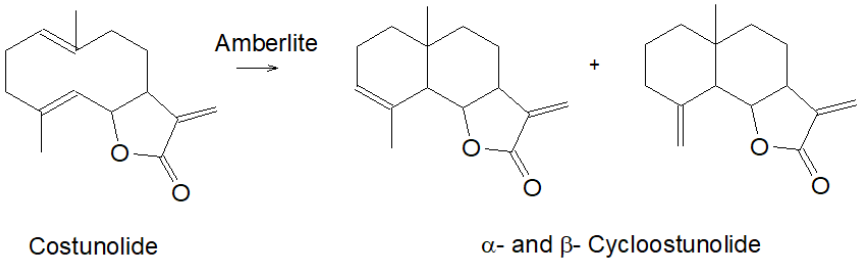

Figure 6. Amberlite-induced transannular cyclization of costunolide, the analogue of parthenolide lacking the epoxide group, into α- and β-cyclocostunolide.

Finally, the acid-catalysed cyclizations of tanaparthin peroxides give rise to the two cyclopentenone-containing lactones present in feverfew, *seco*-tanapartholides A & B. When tanaparthin-α-peroxide was treated with $BF_3 \cdot OEt_2$ ($CHCl_3$, 20ºC; 15 h), *seco*-tanapartholide A was the only product identified (Begley et al, 1989).

After cyclization, parthenolide gives rise mainly to an array of guanolides and xanthanolides. This kind of reaction might occur on the basis of the accelerated degradation of parthenolide when dissolved in citrate buffers at different pHs in the range of 2.4 to 7.2 as reported by Fonseca et al (2003). The resulting end-products are usually naturally occurring sesquiterpene lactones or compounds already present in feverfew like micheliolide, partholide, and *seco*- tanapartholides. In the case of micheliolide, it has been isolated from *Michelia champaca* where it occurs together with parthenolide (Sethi et al, 1984). This plant is reported to be endowed with leishmanicidal, antimicrobial, anti-inflammatory, and antipyretic activities (Takahashi et al, 2004; Khan et al, 2002).

Michael-type additions

The Michael addition is a facile reaction between nucleophiles and activated olefins and alkynes in which the nucleophile adds across a carbon–carbon multiple bonds. The Michael addition benefits from mild reaction conditions, high functional group tolerance, a large host of polymerizable monomers and functional precursors as well as high conversions and favorable reaction rates (Mather et al, 2006). The α,β-unsaturated carbonyl compounds can easily undergo these type of reactions, and α-methylenebutyrolactones fall into this class of compound.

Reaction of sesquiterpene lactones with amino acids and other thiol-containing molecules.

During the early '70s it was already evident to the scientific community that α- methylenebutyrolactones and cyclopentenone moieties exert their pharmacological and toxicological actions mainly by Michael-type additions with cysteine, glutathione, and a number of sulphydryl-bearing cell enzymes (Hall et al, 1979). This idea has been widely corroborated during the last decades and is nowadays accepted after findings that these compounds, including parthenolide, have the capacity to directly bind to the cysteine residue 179 in the activation loop of IKKβ (Kwok et al, 2001).

Dupuis and co-workers (1974) used alantolactone as a model compound to study how the α-methylenebutyrolactones undergoes Michael-type additions with different nucleophilic groups present in amino acids. As a result, the α-methylenebutyrolactone function reacted with the sulphydryl group of cysteine, with the imidazole group of histidine, and the ε-amino group of lysine, but not with the guanido group of arginine, the hydroxyl group of serine, or the thio ether function of methionine (Figures 7 and 8). It is important to point out that the sulphur atom of a thiol is rather more nucleophilic than the oxygen atom of an alcohol.

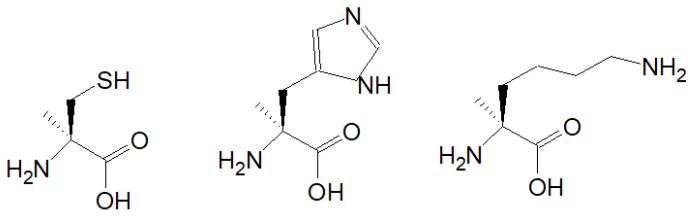

Figure 7. Amino acids with nucleophilic groups that react with α-methylenebutyrolactones (cysteine, histidine, and lysine).

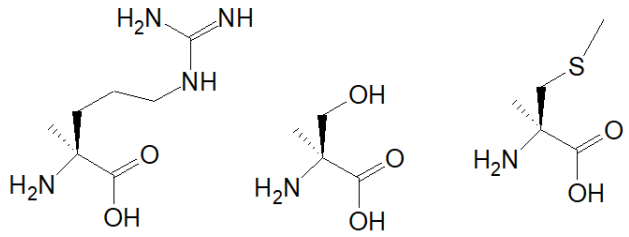

Figure 8. Amino acids with nucleophilic groups that do not react with α-methylenebutyrolactones (arginine, serine, and methionine).

The reaction of α-methylenebutyrolactone-containing sesquiterpene lactones with thiols has been well explored due to its biochemical and pharmacological implications. Sesquiterpene lactones with the cyclopentenone group react very quickly with glutathione (Gly-Cys-γ-Cys) which is present in high concentrations in all living cells, but this reaction is reversible. The α- methylenebutyrolactone is far less reactive with the tripeptide (Schmidt, 1999). In contrast, addition of free cysteine highly favours the exocyclic methylene group (Schmidt, 1997). This property has been exploited by Dolman and co-workers (1992) to set up a specific HPLC-based analyses of α-methylenebutyrolactone-containing sesquiterpene lactones by derivatization with 9-thiomethylanthracene. The thiol containing reagent reacts in a Michael type addition with the α-methylenebutyrolactone of the sesquiterpene lactone and increases the sensitivity, so that routinely nanogram quantities of the lactones can be detected by HPLC and monitoring at 369 nm.

Finally, many naturally occurring sesquiterpene lactones are found to be bound to other α, β unsaturated groups -for example dihydrohelenalin methacrylate- that seem to play no role as a structural element reacting in Michael-type reactions (Wagner et al, 2004)

Reaction of parthenolide with amino acids and other thiol-containing molecules.

Salan (1993) reported that the α-methylene-γ-butyrolactone of parthenolide is not reactive with $–NH_2$ and –OH groups of lysine and serine respectively, and therefore a reaction with such residues do not appear to interfere with the effects of feverfew. In contrast, according to Salan, the cysteine –SH group is very reactive to such a lactone function. In the case of parthenolide the epoxide group allows transannular cyclizations and the generation of a second alkylation site meaning that parthenolide can undergo two Michael-type additions. The reaction is initiated via protonation of the epoxide and may result in the attack of a sulphydryl -usually forming part of an amino acid, peptide or protein- onto the resultant carbocation. In addition, a second sulphydryl -or any other suitable nucleophilic group- can attack the α-methylenebutyrolactone. This chemical feature intensifies the reactivity of parthenolide as an alkylating agent, particularly towards thiols. (Figure 9) (Dewick, 2000).

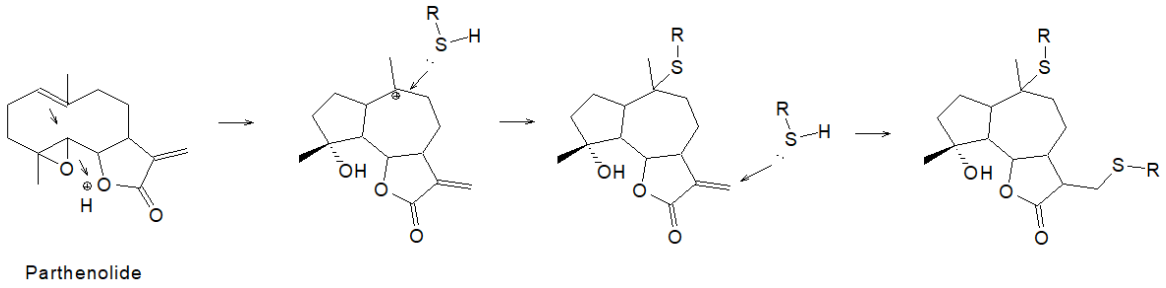

Figure 9. Reactivity of parthenolide with thiols.

The binding of sesquiterpene lactones to albumin, a protein with an important role in the bioavailability, toxicity and pharmacological behaviour of many xenobiotics, is an example of the complexity of the situation when different reactive residues are presented in a macromolecular structure. This protein contains a cysteine and a lysine residue that may react with α, β unsaturated carbonyls in lactones -*i.e*. α-methylenebutyrolactones and cyclopentenones. Franot and co-workers (1993) showed that α-methylene-γ-butyrolactones preferably react with lysine in human albumin, whereas they found that a reaction with cysteine only occurs under reducing conditions. The results of Wagner and co-workers (2004) showed that lactones were able to bind to albumin and other plasma proteins *in vitro* but the extent of the binding to cysteine residues does not explain this phenomenon, but rather the binding to other amino acids, like lysine, as well as non-covalent interactions. The results also showed that the extent of protein binding between individual sesquiterpene lactones was significantly different and in particular parthenolide binds to albumin to a greater extent than the helenalin derivatives do.

Reversibility of the formation of adducts between sesquiterpene lactones and sulphydryl-containing biomolecules.

As mentioned above, the adduct formed by sesquiterpene lactones with a cyclopentenone group reacting with glutathione (Gly-Cys-γ-Cys) is reversible (Schmidt, 1999). The addition of cysteine or other small –SH containing biomolecules to the α-methylenebutyrolactone group has also been investigated and shown to be reversible in several in vitro cell models. These adducts were oxidized by cytochrome P_{450} enzymes, thus releasing the intact lactone inside the cell (Heilmann et al, 2001).

The concept of parthenolide -and similar sesquiterpene lactones- binding to plant protein thiols -or other nucleophilic groups present in biomolecules- after the leaf cells have been disrupted by drying and powdering has been an intriguing hypothesis for many years. Salan (1993) provided the first proof of the presence of such adducts by reversing the Michael-type addition in “old” feverfew samples that did not contain anymore “free” parthenolide according to HPLC-UV analytical protocols. Sequential treatment of this plant material with an oxidant -to convert the putative sulphide into sulfone- and a weak base -to induce elimination- causes the regeneration of substantial amounts of parthenolide. Parthenolide was accompanied by the appearance of michenolide, due to the oxidation (epoxidation) of its endocyclic double bound by the excess of oxidising agent (oxone). Michenolide is however a naturally occurring compound in *Michelia compressa*. Mittra and co-workers (2000) claimed that they could reproduce these results in their laboratory.

Binding of γ- lactones to polysaccharides or polyhydroxypolymers.

Published data indicate that γ- and δ-lactones are able to bind to dispersed hydrated starch. Sorption of lactones is related to van der Waals interactions with polysaccharide molecules, which may lead to the formation of supramolecular structures: after the addition of organic substances into the starch gel, linear molecules of amylose form surrounding spirals and lactones are incorporated into the hydrophobic space between the spirals (Heinemann et al, 2001).

In some cases, these compounds cannot be isolated by extraction from the resulting complex. For example, Misharina and coworkers (2002) showed that a 25% concentration of sorbed γ-decalactone could not be desorbed by ether from gels of corn and potato starches. These authors also showed that the phenomenon of lactone sorption by polysaccharides increases with an increase in their concentration in the gel and lengthening of the alkyl substituent in the lactone molecule. They further proposed that this sorption occurs through a cooperative mechanism, which suggests the formation of supramolecular structures involving hydrated polysaccharide molecules and inclusion complexes with both amylose and lateral chains of amylopectin. The interaction between γ-heptalactone and γ-decalactone molecules with special polysaccharides with amino and sulphate groups (chitosan and carrageenan respectively) has also been studied by the same authors (Misharina et al, 2006).

Degradation of sesquiterpene lactones by the action of radiation and heat.

Ultraviolet (UV) radiation is reported to induce drastic rearrangements in germacranes. Two examples of this are the photoisomerization reported for Germacrene-D, that gives rise to α- and β-bourbonenes (Roberts, 1972), and how the irradiation of germacrene B alcohol, or its corresponding methyl ether, gives rise to three very different products (Roberts and Bryson, 1984) (Figure 10). UV radiation can also alter sesquiterpene lactones in a less extreme way. Bitter guaianolides present in *Chicorium intybus* L. (chicory) are modified by exposure to UV radiation (366nm) prior to extraction of inulin, a valuable food ingredient. The resulting lactones are not bitter as a result of the addition of a molecule of water to the double bound at C_1-C_{10}. The α-methylenebutyrolactone and cyclopentenone groups remained intact (Frey et al, 2002).

Germacrenes can undergo Cope rearrangements under high temperature conditions, irradiation, electronic impact (what is electronic impact?) or a combination of these physical factors. Germacranolides are apparently more resistant to Cope rearrangements because the lactone ring has an important influence on the thermal stability of germacrenes and makes the Cope rearrangement reversible. Cope rearrangements usually give rise to sesquiterpene lactones of the elemane type (Jain et al, 1970; 1971). Costunolide and dihydrocostunolide, that resemble parthenolide both chemically and biosynthetically, undergo Cope rearrangement at 200ºC to give dehydrosaussurealactone and saussurealactone respectively (de Kraker et al, 2000), which are naturally occurring compounds in *Saussurea* species (Roberts, 1972).

According to Cretnik and co-workers (2005) the thermal stability test indicated little degradation of parthenolide at 80°C: after 1 h the parthenolide content in acetonitrile decreased from 10 mg/10mL to 8.7 mg/10mL and after 5 h the content decreased to 8.4 mg/10 mL. This could be due to the strong influence of the lactone ring on the thermal stability of germacrenes (de Kraker et al, 2000) and a good example are some of the apparently unstable epoxidated sesquiterpene lactones found in feverfew such as the crystalline endoperoxide tanaparthin-α- peroxide, which is, in fact, a remarkably stable compound: it only undergoes a smooth, stereospecific, thermal rearrangement in rather extreme conditions (150ºC; C_6D_6; 1.5 h) to give canin, a major natural occurring guaianolide in feverfew (Begley et al, 1989).

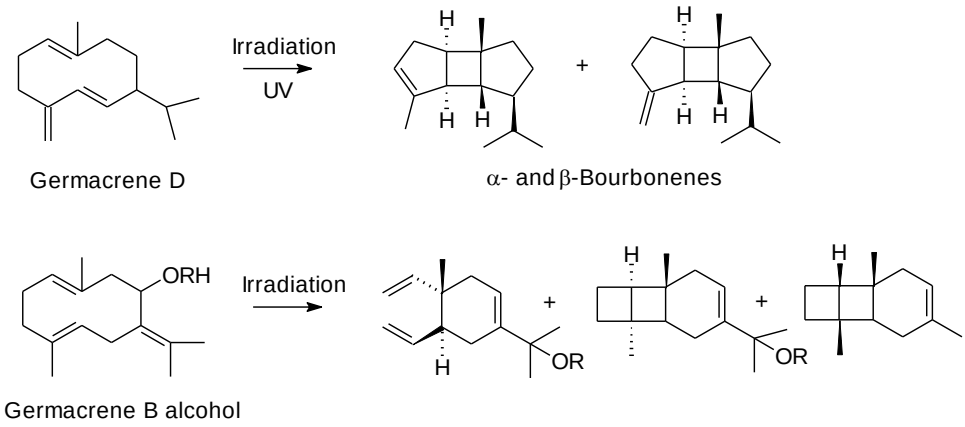

Figure 10. UV-induced rearrangements in germacrenes.

DEGRADATION OF SESQUITERPENE LACTONES BY MICROBIAL ACTION.

Sesquiterpene lactones can be degraded or transformed by microorganisms. The microbial transformation of parthenolide has been studied by Galal and co-workers (1999). Species of *Aspergillus*, *Candida*, *Cimninghamella*, *Gymnascella*, *Lindera*, *Penicillium*, *Rhizopus, Rhodotorula* and *Saccharomyces* were able to convert parthenolide into 11βH-dihydroparthenolide. The reduction of the C_{11}-C_{13} exocyclic double bond is comparable with the type of reactions catalysed by enoate reductases. This is a group of iron-sulphur flavoproteins that are involved in fatty-acid biosynthesis and can be found in many microorganisms (de Kraker et al, 2002) (Figure 11).

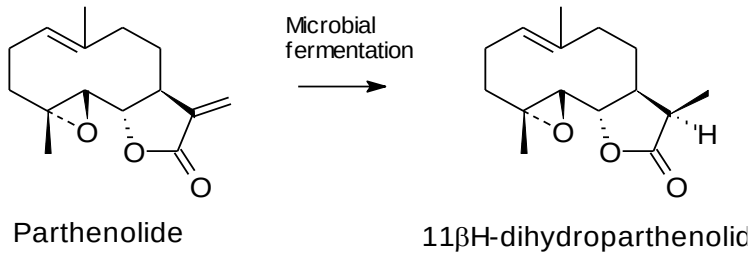

Figure 11. Main product of the microbial degradation of parthenolide.

Other identified minor metabolites were 9β-hydroxy-11βH-dihydroparthenolide, and 14- hydroxy-11βH-dihydroparthenolide that are products of allylic oxidation reactions, which are considered common microbial bioconversions of many unsaturated steroids and terpenoids (Fonken & Johnson, 1972). It was noticed that the microbial reaction proceeds with the retention of the *trans* configuration of the double bond.

The result of the microbial degradation of the α-methylene group is likely to reduce or eliminate the biological activity of sesquiterpene lactones (Ruengeler et al, 1999; Siedle et al, 2004). In addition, the increased polarity of the hydroxy compounds may improve their elimination in whole organisms. Detection of these metabolites by RP-HPLC-UV analyses of plant material may be difficult after a predictable decrease of their elution time and different UV spectral characteristics in comparison to parthenolide and other α-methylenebutyrolactones.

DISCUSSION

The data from the reviewed literature strongly support the idea of a complexation with –SH containing biomolecules, mostly plant proteins. Salan (1993) proved this theory by regenerating parthenolide from old material stored until it did not contain any trace of this compound. Many authors (Knight, 1995; Dewick,

2002, Mittra et al, 2000) also agree that this is the fate of parthenolide, and predictably the rest of sesquiterpene lactones, in dry, powdered feverfew.

The toxicological and pharmacological consequences of such a process have not been directly studied, but indirect data in the literature predict a complete loss of both activities. Michael-type additions imply the reaction of the α-methylene-γ-lactone group of the sesquiterpene lactone with a nucleophilic group giving rise to a covalent bound. This complexation removes the toxicological and pharmacological effects of sesquiterpene lactones in several experimental models (Dupuy et al, 1974; Schmidt et al, 1999).

Other physico-chemical possibilities of degradation can be acid-catalysed cyclizations, and heat or UV induced rearrangements. These reactions show a high degree of stability of the α- methylene-γ-lactone group. Parthenolide may undergo an acid-induced cyclization in aqueous solutions giving rise to a guaianolide-type sesquiterpene lactone - a type of compound that is common in feverfew (Begley et al, 1989). This type of sesquiterpene lactones has been described as a pharmacologically active form of parthenolide (Dewick, 2002). Moreover, it should be recognised that these “degradative processes” are initiated immediately after the harvest of feverfew, during the conditioning of the plant material (drying of leaves). Sorption of lactones with polyhydroxypolymers requires the direct contact of the compound with the polymers in a solution. This process is probably restricted to the formulation of pure parthenolide or parthenolide-rich extracts, which is not the case in formulations consisting of dry plant material mixed with such excipients. Moreover, the phenomenon is highly reversible.

The microbiological degradation of parthenolide usually consists of the selective reduction of the α-methylene group, and we can surmise that this will be accompanied by an important loss of its biological properties.

Finally, formulations of Feverfew used in clinical trials for 4-6 months did not show any toxicity and the reported adverse effects were mild, transient and similar to those reported by the placebo group (Murphy et al, 1998; Johnson et al, 1985; Palevitch et al, 1997) even though a certain degree of degradation would be expected during this time. There are reported differences in the toxicity of old feverfew material and fresh material in that dry encapsulated material is less allergenic than fresh feverfew (Schuller and Cupp, 2000; Mahady et al, 2001).

CONCLUSION

In conclusion, and without discounting a degradation of parthenolide into non-identifiable fragments, the fate of this compound in dry, powdered feverfew is to undergo a covalent binding to plant proteins resulting in a biologically inactive adduct - in accordance with the direct and indirect data found in the literature. This process seems to be virtually unstoppable, and temperature and light do not seem to be playing a significant role under normal storage conditions according to some authors. In the presence of a high level of humidity, parthenolide may undergo an acid-induced cyclisation giving rise to a guaianolide-type sesquiterpene lactone, a class of compound that is commonly found in Feverfew. Microbial degradations are not likely to play an important role if the formulation complies with Pharmacopoeial microbiological quality requirements. The experimental and clinical data in the literature do not report on any increase in the toxicity of stored feverfew. More work needs to be done to devise strategies to prevent loss of parthenolide in final medicinal products for their entire shelf-life (3 years at least) so this active component can be effectively used for the standardisation of feverfew extracts and products.

CONFLICT INTEREST

The author declares no personal or financial conflict of interest related to this work.

AUTHORS CONTRIBUTION

(JMP) Conceptualization, Methodology, Formal analysis, Investigation, Writing, Review & Editing.

REFERENCES

Begley, M.J., Hewlett, M.J., Knight, D.W. (1989) Revised structures for guaianolide α-methylenebutyrolactones from feverfew. Phytochemistry 28(3), 940-943.

Bilia, A.R., Bergonzi, M.C., Mazzi, G., Vincieri, F.F. (2006) Development and stability of semisolid preparations based on a supercritical CO_2 Arnica extract. Journal of Pharmaceutical and Biomedical Analysis 41, 449–454.

Brown, H.C., Brewster, J.H., Shechter, H. (1954) An interpretation of the chemical behaviour of five- and six-membered ring compounds. Journal of the American Chemical Society 76, 467-474.

Cane, D.E. (1999) Sesquiterpene biosynthesis: cyclization mechanisms. In Cane, D.E. (Ed) Comprehensive Natural Products Chemistry: Isoprenoids including caretenoids and steroids Vol 2, Elsevier, Oxford (UK), pp 155-200.

Castaneda-Acosta, J., Fischer, N.H., Vargas, D. (1993) Biomimetic transformations of parthenolide. Journal of Natural Products, 56(1), 90-98.

Cretnik, L., Skerget, M., Knez, Z. (2005) Separation of parthenolide from feverfew: performance of conventional and high-pressure extraction techniques. Separation and Purification Technology 41, 13–20.

de Kraker, J-W., Franssen, M.C.R., Joerinkm M., de Groot, A., Bouwmeester, H.J. (2002) Biosynthesis of costunolide, dihydrocostunolide, and leucodin. Demonstration of cytochrome P450-catalyzed formation of the lactone ring present in sesquiterpene lactones of chicory. Plant Physiology 129, 257–268.

Dewick, P.M. (2002) Medicinal Natural Products. John Wiley & Sons, London, pp 191-196.

Dolman, D.M., Knight, D.W., Salan, U., Toplis, D. (1992) A quantitative method for the estimation of parthenolide and other Sesquiterpene lactones containing α-methylenebutyrolactone functions present in feverfew. Phytochemical Analysis 3, 26- 31.

Dupuis, G., Mitchell, J.C., Towers, G.H.N. (1974) Reaction of alantolactone, an allergenic sesquiterpene lactone, with some amino acids. Resultant loss of immunologic reactivity. Canadian Journal of Biochemistry 52(7), 575-581.

Fonken, G.S., Johnson, R.A. (1972) Chemical oxidations with microorganisms. In: Belew, J.S. (ed) Oxidation in organic chemistry, vol 2. Marcel Dekker, New York, pp 185–212.

Fonseca, J.M., Rushing, J.W., Thomas, R.L., Riley, M.B., Rajapakse, N.C. (2003) Post-production conditions affect stability of parthenolide in feverfew. Horticultural Science 38, 1294.

Fonseca, J.M., Rushing, J.W., Thomas, R.L., Riley, M.B., and Rajapakse, N.C. (2007). Post-Production Stability of Parthenolide in Feverfew (Tanacetum parthenium). Journal of Herbs, Spices & Medicinal Plants 12, 139-152.

Franot, C., Benezra, C., Lepoittevin, J.P. (1993) Synthesis and interaction studies of ^{13}C labelled lactone derivatives with a model protein using ^{14}C NMR. Bioorganic and Medicinal Chemistry 1, 389-397.

Frey, U., Claude, J., Crelier, S., Juillerat, M.A. (2002) UV degradation of sesquiterpene lactones in chicory extracts: kinetics and identification of reaction products by HPLC- MS. Chimia 56, 292-293.

Galal, A.M., Ibrahim, A.S., Mossa, J.S., El-Feraly, F.S. (1999) Microbial transformation of parthenolide. Phytochemistry 51, 761-765.

Groenewegen, W.A., Knight, D.W., Heptinstall, S. (1992) Progress in the medicinal chemistry of parthenolide. In Ellis GP and Luscombe DK (Eds) Progress on Medicinal Chemistry Vol 29, Elsevier, Amsterdam, 217-238.

Hall, I.H., Lee, K.H., Starnes, C.O., Sumida, Y., Wu, R.Y., Waddell, T.G., Cochran, J.W., Gerhart, K.G. (1979) Anti-inflammatory activity of sesquiterpene lactones and related compounds. Journal of Pharmaceutical Sciences 68(5), 537-542

Heilmann, J., Wasescha, M.R., Schmidt, T.J. (2001) The influence of glutathione and cysteine levels on the cytotoxicity of helenanolide type sesquiterpene lactones against KB cells. Bioorganic & Medicinal Chemistry 9, 2189–2194.

Heinemann, C., Conde-Petit, B., Nuessli, J., Escher, F. (2001) Evidence of starch inclusion complexation with lactones. Journal of Agricultural and Food Chemistry 49(3), 1370– 1376.

Heptinstall, S., Awang, D.V., Dawson, B.A., Kindack, D., Knight, D.W., May, J. (1992) Parthenolide content and bioactivity of feverfew (Tanacetum parthenium (L.) Schultz- Bip.). Estimation of commercial and authenticated feverfew products. Journal of Pharmacy and Pharmacology 44(5), 391-395.

Heptinstall, S., Awang, D.V.C. (1998) Feverfew: a review of its history, its biological and medicinal properties, and the status of commercial preparations of the herb. In ACS Symposium Series Vol 691 (Phytomedicines of Europe), American Chemical Society, pp 158-175.

Hoffmann, J.J., Torrance, S.J., Wiedhopf, R.M., Cole. J,R. (1977) Cytotoxic agents from Michelia champaca and Talauma ovata: parthenolide and costunolide Journal of Pharmaceutical Sciences 66, 883-884.

Jain, T.C., Banks, C.M., McCloskey, J.E. (1970) Dehydrosaussurea lactone and reversibility in the germacranolide-Cope reaction. Tetrahedron Letters 11, 841–844.

Jain, T.C., McCloskey, J.E., Banks, C.M. (1971) Germacranolide-Cope reaction under electron-impact. Organic Mass Spectrometry 5, 751–755..

Johnson, E.S., Hylands, P.J., Hylands, D.M. (1982) Eur Pat EP 98401 (GB Pat, 1982, 82/14572).

Johnson, E.S., Kadam, N.P., Hylands, D.M., Hylands, P.J. (1985) Efficacy of feverfew as prophylactic treatment of migraine. British Medical Journal 291, 569-573.

Khan, M.R., Kihara, M., Omoloso, A.D. (2002) Antimicrobial activity of Michelia champaca. Fitoterapia 73(7-8), 744-748.

Knight DW. 1995. Feverfew: chemistry and biological activity. Natural Products Reports 12(3), 271-276.

Kwok, B.H.B., Koh, B., Ndubuisi, M.I., Elofsson, M., Crews, C.M. (2001) The anti- inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase. Chemical Biology 8, 759–766.

Mahady, G.B., Fong, H.H.S., Farnsworth, N.R. (2001) Botanical dietary supplements: quality, safety and efficacy, Swets & Zeitlinger, Lisse (The Netherlands), pp 87-95.

Majdi, M., Liu, Q., Karimzadeh, G., Malboobi, M.A., Beekwilder, J., Cankar, K., Vos, R.D., Todorović, S., Simonović, A., and Bouwmeester, H. (2011). Biosynthesis and localization of parthenolide in glandular trichomes of feverfew (Tanacetum parthenium L. Schulz Bip.). Phytochemistry 72, 1739-1750.

Majdi, M., Charnikhova, T. & Bouwmeester, H. (2013) Genetical, developmental and spatial factors influencing parthenolide and its precursor costunolide in feverfew (*Tanacetum parthenium* L. Schulz Bip.). *Industrial Crops and Products*, 47, 270-276.

Marete, E. N., Jacquier, J.-C. & O'Riordan, D. (2013) Effect of processing temperature on the stability of parthenolide in acidified feverfew infusions. Food Research International, 50(2), 593-596.

Marete, E. N., Jacquier, J.-C. & O'Riordan, D. (2011) Feverfew as a source of bioactives for functional foods: Storage stability in model beverages. Journal of Functional Foods, 3(1), 38-43.

Mather, B.D., Viswanathan, K., Miller, K.M., Long, T.E. (2006) Michael addition reactions in macromolecular design for emerging technologies. Progress in Polymer Sciences 31, 487–531

Misharina, T.A., Terenina, M.B., Krikunova, N.I., Kalinchenko, M.A. (2006) Sorption of components from a mixture of odorants by polysaccharides of starch, chitosan, and carrageenan. Applied Biochemistry and Microbiology 42(1), 111–115.

Misharina, T.A., Terenina, M.B., Krikunova, N.I. (2002) Binding of Lactones by Polysaccharides of Corn and Potato Starches. Applied Biochemistry and Microbiology 38(6), 583–587.

Mittra, S., Datta, A., Singh, S.K., Singh, A. (2000) 5-Hydroxytriptamine-inhibiting property of Feverfew: role of parthenolide content. Acta Pharmacologica Sinica 21(12), 1116- 1114.

Murphy, J.J., Heptinstall, S., Mitchell, J.R. (1988) Randomised double-blind placebo- controlled trial of feverfew in migraine prevention. Lancet 2, 189-192.

Palevitch, D., Earon, G., Carasso, R. (1997) Feverfew (*Tanacetum parthenium*) as a prophylactic treatment for migraine: a double-blind placebo-controlled study. Phytotherapy Research 11(7), 508-511.

Piet, D.P., Schrijvers, R., Franssen, M.C.R., de Groot, Ae. (1995) Biotransformation of germacrene epoxides by Cichorium intybus L. Tetrahedron 51, 6303–6314.

Roberts, J.S., Bryson, I. (1984) Sesquiterpenoids. Natural Products Reports. 105-169.

Roberts, J.S. (1972) The sesquiterpenes. In Newman AA Ed. Chemistry of terpenes and terpenoids. Academic Press, London (UK) pp 88-146.

Ruengeler, P., Castro, V., Mora, G., Goren, N., Vichnewski, W., Pahl, H.L. 1999. Inhibition of transcription factor NF-kappaB by sesquiterpene lactones: a proposed molecular mechanism of action. Bioorganic and Medicinal Chemistry 7, 2343-2352.

Salan, U. (1993) Chemistry of Feverfew. Dissertation. Univ. of Nottingham, 135-168.

Schmidt, T.J. (1997) Helenanolide-type sesquiterpene lactones III. Rates and stereochemistry in the reaction of helenalin and related helenanolides with sulfhydryl containing biomolecules. Bioorganic and Medicinal Chemistry 5, 645-653.

Schmidt, T.J., Lyß, G., Pahl, H.L., Merfort, I. (1999) Helenanolide type sesquiterpene lactones. Part 5: the role of glutathione addition under physiological conditions. Bioorganic and Medicinal Chemistry 7, 2849-2855.

Schmidt, T.J., Matthiesen, U., Willuhn, G. (2000) On the stability of sesquiterpene lactones in the official Arnica Tincture of the German Pharmacopoeia. Planta Medica 66, 678- 681.

Schuller, B., Cupp, M.J. (2000) Feverfew. In Cupp MJ (Ed.) Forensic Science: Toxicology and Clinical Pharmacology of Herbal Products, Humana Press, Totowa (NJ, USA) pp 95-105.

Sethi, V.K., Thappa, R.K., Dhar, K.L., Atal, C.K. (1984) Constituents of Michelia champaca and Lewis acid catalyzed transformations of parthenolide into guaianolides. Planta Medica 50(4), 364.

Siedle, B., Garcia-Pineres, A.J., Murillo, R., Schulte-Monting, J., Castro, V., Rungeler, P., Klaas, C.A., Da Costa, F.B., Kisiel, W., Merfort, I. (2004) Quantitative structure-activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-κB. Journal of Medicinal Chemistry 47, 6042-6054.

Takahashi, M., Fuchino, H., Satake, M., Agatsuma, Y., Sekita, S. (2004) In vitro screening of leishmanicidal activity in myanmar timber extracts. Biological and Pharmaceutical Bulletin 27(6), 921-925.

Tanko, H.M., Carrier, D.J., Sokhansanj, S., Crowe, T.G. (2003) Effects of drying temperature and storage on parthenolide concentration of feverfew (Tanacetum parthenium L.) leaves. Journal of Nutraceuticals, Functional & Medical Foods 4(1), 27-37.

Wagner, S., Kratz, F., Merfort, I. (2004) In vitro behaviour of sesquiterpene lactones and sesquiterpene lactone-containing plant preparations in human blood, plasma and human serum albumin solutions. Planta medica 70(3), 227-233.

Wang, M. & Li, Q. (2015) Parthenolide could become a promising and stable drug with anti-inflammatory effects. Natural Product Research, 29(12), 1092-1101.

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Review

Acne vulgaris: the skin microbiome, antibiotics and whether natural products could be considered a suitable alternative treatment?

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Abstract

Acne vulgaris is a common, chronic inflammatory skin disease manifested as inflammatory and non-inflammatory lesions typically associated with *Cutibacterium acnes.* However, its pathogenesis is not fully understood nor is the complexity of the skin microbiome and how it contributes to the development of acne. Whilst acne is not a typical bacterial infection, antibiotics have been the mainstay of treatment for over 50 years.

Now, with the development of multi-drug resistant organisms and the emergence of resistant *C. acnes* strains; the question is are antibiotics still an appropriate treatment method or could natural products provide a suitable alternative? Research into alternative treatments is a growing field due to the increase in resistant organisms, there is a multitude of research into natural products due to their antimicrobial potential and the multiple mechanisms of action.

Melaleuca alternifolia is a key natural product of interest in the treatment of acne due to its documented use throughout history and its prevalence in over-the-counter treatments. Green Tea is a more recent natural product of interest due to its composition of polyphenols, which give rise to both antimicrobial and anti-inflammatory properties. However, research also suggests that a synergistic approach of natural products may be the way forward.

Keywords Acne vulgaris, skin microbiome, antibiotics, natural products.

INTRODUCCION

Acne vulgaris is a common, chronic inflammatory skin disease, ranking second as the most common dermatological condition (Xu and Li, 2019). It's prevalent in approximately 80% of all 11-30 years olds (Owen *et al*, 2017); despite its prevalence in young adults and adolescents it may persist into adulthood largely affecting females (Fox *et al*, 2016). This disease affects the pilosebaceous units of the skin resulting in the characteristic seborrhoea, non-inflammatory lesions (comedones) and inflammatory lesions (papules, pustules, nodules and cysts) as well as scarring, erythema and hyperpigmentation (Fox *et al*, 2016; Walsh, Efthimiou and Dréno, 2016). Dependent on the number and type of lesions, acne diagnosis is classified as mild, moderate or severe (Farrah and Tan, 2016). Lesions generally present in areas with a high proportion of sebaceous glands, such as, face, chest, upper back and upper arms (Fox *et al*, 2016; Owen *et al*, 2017; Walsh, Efthimiou and Dréno, 2016). Despite acne being a non-life-threatening skin condition (Greener, 2016), it has been associated with a negative psychological impact; although this may not always correlate with the clinical severity, it is often associated with self-perceived severity (Agnew, Leach and Segal, 2014). The psychological impact is considered substantial due to reports identifying an increased risk of acne patients developing insomnia, depression and anxiety (Trivedi *et al*, 2018); whilst also causing psychosocial symptoms such as embarrassment, emotional stress and maintaining relationships and friendships. Majority of the adverse psychological and psychosocial effects can be contributed to the discomfort, scarring and permanent disfigurement patients with severe acne suffer from (Fox *et al*, 2016). Therefore, effective treatment of acne is of great importance.

PATHOGENESIS OF *ACNE VULGARIS*

Pathogenesis of acne is a complex (Greener, 2016), multifactorial process that is still not fully understood (Dessinioti and Katsambas, 2017; Owen *et al*, 2017). It is thought to involve four major factors: a) enhanced sebum production, b) abnormal follicular hyperkeratinisation, c) an anaerobic, lipid-rich environment allowing *Cutibacterium acnes* to proliferate, d) host-inflammatory response and inflammation within the area (Farrah and Tan, 2016; Fox *et al*, 2016; Owen *et al*, 2017; Xu and Li, 2019; Walsh, Efthimiou and Dréno, 2016), as shown in Figure 1.

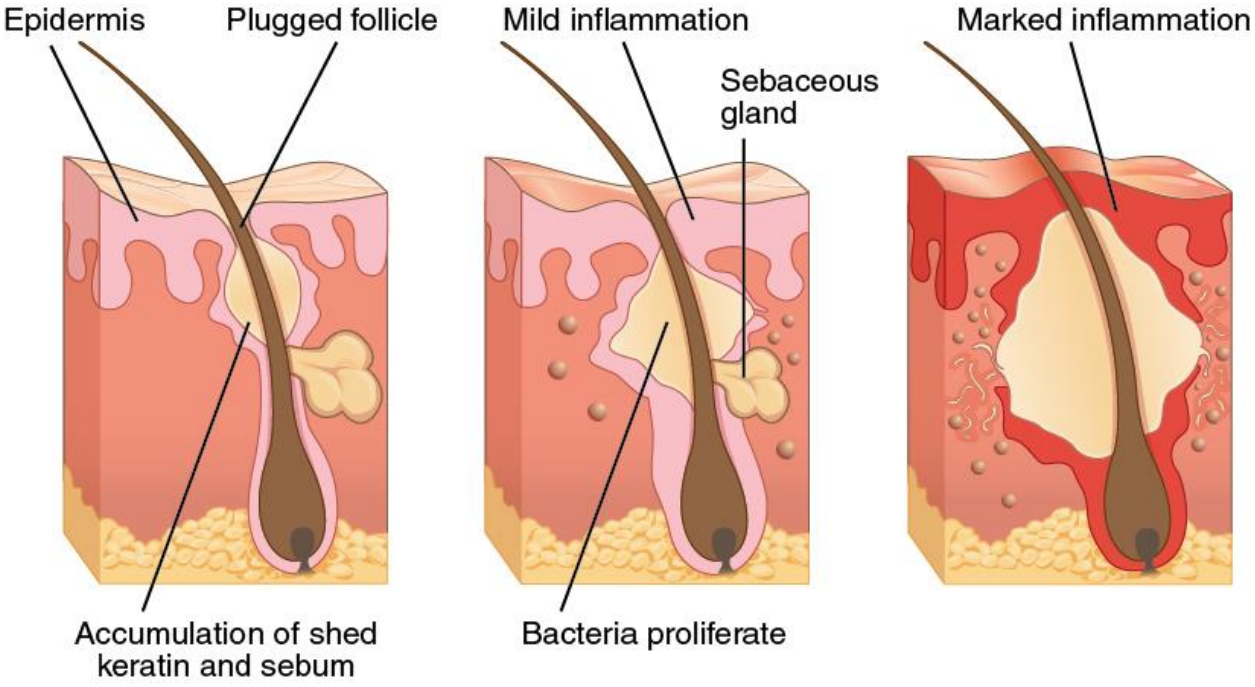

Figure 1 | A detailed image depicting the formation of acne (Betts *et al.*, 2013) (Creative Commons — Attribution 4.0 International — CC BY 4.0).

Although sebum has antibacterial functions, *C. acnes* hydrolyses triglycerides present in the secretions, releasing short chain free fatty acids such as propionic acid and porphyrins promoting bacterial colonisation of these glands (Grice and Segre, 2011; Dréno, 2017; Rocha and Bagatin, 2017; O'Neill and Gallo, 2018; Xu and Li, 2019). Porphyrins can generate reactive oxygen species, which have the ability to induce inflammation in keratinocytes resulting in the production of acne lesions via *C. acnes* binding to toll-like-receptor- 2(TLR-2) and TLR-4. *P. acnes* also has the capability to activate the classical and alternative complement pathways increasing the vascular permeability (Dréno, 2017; O'Neill and Gallo, 2018; Xu and Li, 2019; Platsidaki and Dessinioti, 2018).

Acne tends to present in adolescents as during puberty androgen hormones lead to an increased production of sebum which in turn promotes a high colonisation of *C. acnes* within the pilosebaceous follicle (Rocha and Bagatin, 2017; Platsidaki and Dessinioti, 2018). Thus, confirming that the microbial dysbiosis in acne could be attributed to the exacerbation of androgen-mediated seborrhoea and dysborhea (O'Neill and Gallo, 2018).

THE SKIN MICROBIOME

The skin microbiome is a complex ecological system (Christensen and Brüggemann, 2014; O'Neill and Gallo, 2018) that is unique to an individual (Christensen *et al*., 2016; Dréno, 2017); therefore, it is difficult to define a "healthy" skin microbiome (O'Neill and Gallo, 2018). The constituents of the microbial community include bacteria, viruses, fungi, protozoa and arthropods (Schommer and Gallo, 2013; Christensen and Brüggemann, 2014). Under normal physiological conditions, this ecosystem maintains homeostasis between the resident microbiome, transient microbes and the host when it is continuously being influenced by external and internal factors (Dréno *et al*., 2018; Schommer and Gallo, 2013; Dréno, 2017). Skin microbiome homeostasis is essential to healthy skin activity as it results in the inhibition of bacterial hyperproliferation of pathogenic commensals that are involved in various skin conditions (Xu and Li, 2019; Rocha and Bagatin, 2017).

When it comes to investigation of the skin microbiome, majority of research is regarding bacteria, this is possibly due to bacteria being the most prevalent microorganism to colonise the skin (Schommer and Gallo, 2013). There are four major phyla that dominate the skin: Actinobacteria, Proteobacteria, Bacteroidetes and Firmicutes (O'Neill and Gallo, 2018). Interestingly, these reflect the phyla that dominate the intestinal microbiome leading to the hypothesised skin-gut axis and why a high glycemic index diet is considered as a factor in the development of acne (Thursby and Juge, 2017; Salem, Ramser, Isham and Ghannoum, 2018). From these phyla more than 60% of the bacterial species belong to the genera *Staphylococcus*, *Corynebacterium* and *Propionibacterium* (Rocha and Bagatin, 2017. The most significant in the development of acne being that of *Cutibacterium acnes* and *Staphylococcus epidermidis* (Fox *et al*, 2016; Christensen *et al*., 2016).

Little is known regarding the skin virome possibly due to issues in the amplification of viruses in cell culture or limited antigenic and serological cross-reactivity; however, there is a controversial hypothesis that suggests pathogenic viruses such as the human papillomavirus (HPV) are part of the normal skin microbiome although this has not been fully elucidated (Schommer and Gallo, 2013).

Although, it is known that fungi are a constituent of a healthy skin microbiome, there is little information available regarding their ecological interaction in the state of health (Schommer and Gallo, 2013). However, it is known that *Malassezia spp.* have been detected in the follicles of patients with acne due to their favour of sebum lipids and was correlated with that of inflammatory acne (Christensen and Brüggemann, 2014). This could potentially indicate that *Malassezia* spp. could have a role in the development in acne although further investigations are needed alongside more research into the other constituents of the skin microbiome. Like the *Malassezia* spp., *Demodex folliculorum* favour lipid sebum and are found to inhabit the follicles (Schommer and Gallo, 2013).

Cutibacterium acnes

Cutibacterium acnes formally known as *Propionibacterium acnes* is an aerotolerant, anaerobic gram-negative bacilli which colonises the pilosebaceous follicles (Dréno *et al*., 2018; Esmael *et al*., 2019); therefore, it is more prevalent in areas which contain a large volume of these follicles such as the face, upper limbs and torso (Xu and Li, 2019). It has been proven that *P. acnes* is one of the most established

bacteria of the skin microbiome in both those with “healthy” skin and those with a form of acne (Dessinioti and Katsambas, 2017), although its presence is limited to the pilosebaceous follicles (Dréno *et al*., 2018; Fox *et al*, 2016).

A study by Fitz-Gibbon *et al* (2013), identified that certain *C. acnes* strains as shown in Table 1, were heightened in acne patients whilst others were predominantly found in individuals with a “healthy” skin microbiome. This is further clarified by Kwon, Yoon, Park and Suh, (2013) noted that although phylotype distribution was similar in skin surface and comedone lesion there was an increase in phylotype 1A-1 and a decrease in phylotype 1b and II in papules and pustules. Therefore, it was considered that phylotype 1A-1 was strongly associated with acne whilst phylotype II was associated with a “healthy” skin microbiome. In contrast to this an observational prospective study by Paugam *et al*, (2017) discovered no difference in the distribution of phylotypes between patients with mild and severe acne despite the phylotype 1A-1 being the most prevalent in both populations.

Despite being connected with the inflammatory condition acne, *C. acnes* also works to maintain the balance of the skin microbiome. It has the ability to limit the proliferation of pathogenic transient microbes, such as *Staphylococcus aureus* and *Streptococcus pyogenes*; via the hydrolysis of triglycerides in sebum releasing short-chain fatty acids aforementioned, which have antimicrobial properties in maintaining the acidic pH of the skin surface (Dréno, 2017; Xu and Li, 2019; O’Neill and Gallo, 2018). However, this then favours the growth of coagulase-negative *Staphylococci* and *Corynebacterial* growth (Grice and Segre, 2011).

Table 1 | A table identifying the different Clade formats against the ribotype and whether the strains are present in acne or healthy skin; with Clade 1 based on whole-genome sequence comparison whilst Clade 2 is based on Belfast *e*MLST and Clade 3 based on Aarhus MLST. (*e*MST expanded multi-locus sequence typing, MLST multi-locus sequence typing) (Xu and Li, 2019).

Clade 1	Clade 2	Clade 3	Ribotype	Acne	Healthy Skin
IA-1	IA1	I-1a	RT 1	Yes	Yes
IA-2	IA1	I-1a	RT4, RT5	Yes	No
IB-1	IA1	I-1b	RT8	Yes	No
IB-2	IA2	I-1a	RT3	Yes	Yes
IB-3	IB	I-2	RT1	Yes	Yes
IC	IC	NA	RT5	Yes	No
II	II	II	RT2, RT6	No	Yes
III	III	III	NA	No	No

Staphylococcus epidermidis

S. epidermidis is a coagulase-negative, facultative anaerobe coccus, which is a major skin commensal which occupies more than 27% of the total bacteria population (Christensen et al., 2016; Dréno et al., 2018). The successful colonisation of *S. epidermidis* is a result of its commensal lifestyle, which favours traits conferring persistency over aggressive host-damaging properties; with its low cytotoxicity and ability to evade host defences ensuring a low host immune response (Christensen and Brüggemann, 2014). Despite is prevalence it is not considered to be a causative agent of acne; in fact, it is suggested that there is an alliance between *S. epidermidis* and the host to keep potential transient pathogens from colonising the skin (Christensen and Brüggemann, 2014; Dréno, 2017; Rocha and Bagatin, 2018). Despite being a commensal of the skin, *S. epidermidis* has the ability to act as an opportunistic pathogen should it breach the skin surface and enter the bloodstream (Christensen and Brüggemann, 2014).

Antagonistic interactions between *S. epidermidis* and *C. acnes* are considered a common occurrence due to their close proximity to each other in the skin microbiome, although, the relevance in skin health and disease is greatly unknown (Christensen et al., 2016). However, a study by Christensen et al (2016) suggested that due to interspecies interaction there could a potential of disrupting the homeostasis of the

skin microbiome due to the various inhibition mechanisms that *S. epidermidis* possesses in relation to C. acnes. Although this study could not confirm the relevance of these interactions in skin disorders.

Antibiotic Treatment

Antibiotics have been the mainstay of acne treatment for over 50 years even though acne is not a bacterial infection but could be considered an inflammatory reaction to the skin microbiome (Humphrey, 2012; Greener, 2016; Walsh, Efthimiou and Dréno, 2016). Antibiotic treatment in acne involves the use of oral and or topical antibiotics intended to reduce the quantity of *P. acnes* colonising the pilosebaceous follicle, as it is understood that *P. acnes* is the principal bacteria involved in the pathogenesis of acne lesions (Farrah and Tan, 2016; Humphrey, 2012). However, some antibiotics specifically cyclines have significant anti-inflammatory properties which inhibit the production of *C. acnes* associated inflammatory mediators; suggesting that antibiotics have a more important role than the antimicrobial activity (Walsh, Efthimiou and Dréno, 2016; Humphrey, 2012; Greener, 2016). Although, this has not yet been reported in vivo, the suggestion is hypothesised based on the copious in vitro data which identifies that antibiotics have activities unrelated to eradicating bacteria (Walsh, Efthimiou and Dréno, 2016).

Whilst reducing the quantity of *P. acnes* may seem to improve acne, it may not resolve or cure the condition (Farrah and Tan, 2016). In fact, antibiotics can cause dysbiosis of the microbiome, potentially encouraging the emergence of pathogenic transient bacteria such as S. aureus, Methicillin-Resistant S. aureus (MRSA) and other resistant bacteria (Greener, 2016). This can potentially lead to the host developing infections from pathogenic and highly resistant organisms should they breach the skin barrier and defence mechanisms. With topical antibiotics this is limited to the area treated; however with oral antibiotics the body as a whole may be affected (Walsh, Efthimiou and Dréno, 2016).

ANTIMICROBIAL RESISTANCE IN ACNE

According to the O'Neill Report (2016), antimicrobial resistance is a major threat to public health worldwide and is still on the rise, impacted by the overuse of antimicrobials, which in turn has increased the rate in which resistance is developing. It is estimated that by 2050, 10 million lives per year are at risk due to the development of drug-resistant infections. It currently stands at 700,000 deaths per year, which is an increase of 1430%. The use of antibiotics in acne is vastly contributing to this risk with the prevalence of resistant strains of *P. acnes* being observed over the years (Humphrey, 2012).

There was no evidence of antibiotic resistant *C. acnes* in the skin microbiome of over 1000 patients in 1976 (Leyden, 1976), yet, by 1979 Crawford and colleagues discovered the first indications of resistance, specifically to topical erythromycin and clindamycin followed by tetracycline. Since this development, antibiotic resistance in acne has continued to rise globally with incidence reaching of 20% in 1978 reaching 62% in 1996 (Walsh, Efthimiou and Dréno, 2016). It is thought that resistant strains of *C. acnes* can emerge relatively quickly as a result of chromosomal point mutations mainly in the 23S rRNA gene for macrolide resistance and 16S rRNA for tetracycline resistance (Dréno et al., 2018; Greener, 2016; Humphrey, 2012). However, *C. acnes* is naturally a relatively resilient organism with variants having the ability to withstand antibiotic treatment without resistance (Walsh, Efthimiou and Dréno, 2016); therefore, this could favour some of the conflicting evidence that resistant strains remain after treatment (Farrah and Tan, 2016).

Evidence suggests that *C. acnes* colonies in the pilosebaceous follicle develop macrocolonies resulting in the production of large biofilms, which are notoriously difficult to eradicate due to their intrinsic properties of increased tolerance to antibiotics (Hall and Mah, 2017; Dréno et al., 2018). It is suggested that planktonic and sessile cells do not share identical transcriptomes or proteomes, therefore, indicating that there were phenotypic differences between the two (Resch, Rosenstein, Nerz and Gotz, 2005; Hall and Mah, 2017). This could be confirmed by the work of Jahns et al (2012), who found no qualitative differences between *C. acnes* biofilms in acne and controls; instead, it was inferred that it was phenotypic changes rather than genetic changes that accounted for the pathogenic role of *C. acnes* in acne. This confers the data that identifies aforementioned phylotype 1A-1 as being highly associated with erythromycin and clindamycin resistant *P. acnes* strains (Dréno et al., 2018).

ANTIBIOTIC GUIDELINES IN ACNE

Despite acne not being a typical bacterial infection, antibiotics are still considered to have a role in the treatment of moderate to severe acne. However, there has been guidelines put in place to ensure that effective usage of this important medication as shown in Table 2.

Table 2 | A table to identify the numerous guidelines by the Global Alliance to Improve Outcomes in Acne, The European-Evidence-based Guidelines for the Treatment of Acne and the American Academy of Dermatology that have been put into place in order to limit the further development of antibiotic resistance in acne (Humphrey, 2012; Farrah and Tan, 2016; Walsh, Efthimiou and Dréno, 2016).

	Guidelines around Antibiotic Treatment in Acne
1	Avoid Topical or Oral Antibiotics as monotherapy
2	Avoid topical or Oral Antibiotics as Maintenance Therapy
3	Limit the duration of antibiotic use (3 months or less)
4	The use of oral antibiotics only in moderate & moderately severe acne
5	Oral Antibiotics only used as Induction Therapy
6	Use of Oral Erythromycin & other macrolides restricted to cases where cyclines are contraindicated or not tolerated
7	Oral Clindamycin is not recommended for acne treatment
8	Mild to Moderate acne treatment should be combined with a non-antimicrobial topical agent
9	Topical agents such as retinoids or Benzoyl peroxide added to the treatment regime

These current guidelines in place come from various institutions, such as Global Alliance to Improve Outcomes in Acne, The European-Evidence-based Guidelines for the Treatment of Acne and the American Academy of Dermatology in order to minimise the effect of antibiotic resistance in acne. It is thought that these guidelines could limit the transfer of resistant genes to potentially pathogenic bacteria, which could present difficult clinical challenges especially if the resistant genes were relative to the first line systemic agents for the treatment of MRSA (minocycline, trimethoprim-sulfamethoxazole, clindamycin and doxycycline) [Humphrey, 2012; Farrah and Tan, 2016; Walsh, Efthimiou and Dréno, 2016].

However, even though these guidelines have been put in place researchers have found that treatment does not always follow the recommendations and guidelines in place (Greener, 2016). The analysis of 928 acne patients by Whitehouse et al in 2016, discovered that on average the patients received oral antibiotics for 6.5 months compared to the recommended time of 3 months or less: thus, resulting in the lengthy exposure of antibiotics allowing the virulent strains of *P. acnes* to potentiate resistance and potentially confer to other bacteria colonising the skin microbiome.

There are some countries which have limited the use of antibiotics to treat acne which has resulted in low resistance levels being identified; emphasising the need for the global economy to reduce their use in relation to this chronic condition (Ross et al, 2003; Sardana and Garg, 2014). With the levels of resistance correlating to the levels of antibiotics use (Walsh, Efthimiou and Dréno, 2016; Humphrey, 2012) and the risk of further antibiotic resistance in mind, the consequence of causing dysbiosis in the microbiome of an individual attempting to treat a non-infectious *P. acnes* biofilm; the question becomes whether antibiotics are really a suitable treatment or are there alternative treatment methods available?

COMPLEMENTARY AND ALTERNATIVE THERAPIES

The term complementary and alternative medicine (CAM) therapies covers a vast array of products and procedures that can be of use in the treatment of acne; however, the specific area of interest here is botanicals. The use of plant extracts and herbs as a means of treatment originated in ancient Egypt and Greece from as early as 4500BC to between 500 and 400BC retrospectively (Elshafie and Camele, 2017). Due to the development of multi-drug resistant organisms, the interest in botanicals as alternative treatment methods has greatly increased possibly due to their added benefit of possessing several modes of action due to their chemical composition (Fox et al, 2016).

One of the main categories of botanicals researched is essential oils (EO). EO are volatile secondary metabolites produced by various plant components, with around 300 EO available commercially (Wińska et al., 2019). They are comprised of complex organic chemical arrangements which includes compounds

such as alcohols, aldehydes, terpenes and phenols, which are known to give rise to their various antimicrobial properties with some plants even possessing anti-inflammatory properties (Fox et al, 2016; Tariq et al, 2019). Due to the perception as being “natural” rather than allopathic medicines they are believed to be safe and non-toxic due to their longstanding usage over many centuries; however, this is a misconception, there are many scientific studies which have highlighted that EO have the ability to produce adverse effects with the most common being that of skin irritation or allergic contact dermatitis due to their topical use (Orchard and van Vuuren, 2017; Winkelman, 2018).

Tea Tree oil

M. alternifolia (Maiden & Betche) Cheel of the family Myrtaceae -commonly known as Tea Tree oil (TTO)- is one of the most well-recognised EO due to its medicinal use over a number of decades; it is the product of the Melaleuca alternifolia shrub endemic to Australia and was traditionally used by aboriginals in the treatment of cold and wounds (Plant, Dinh, Argo and Shah, 2019).

Due to the quantity of patients that self-treat acne with TTO and its presence in several over the counter acne products an international standard has been formulated for its use (Winkelman, 2018; Fox et al, 2016).

There has been ample research regarding the effect of TTO on acne due to its constituents. As TTO is composed of terpene hydrocarbons, monoterpenes, sesquiterpenes and associated alcohols, with the major component being that of terpinen-4-ol (Figure 2), which should comprise more than 30% of the TTO concentration and 1,8-cineole less than 15% concentration according to the International Organisation for Standardisaton (Lee et al, 2013). This is due to 1,8-cineole being considered as the undesirable allergen in TTO, potentially responsible for the skin irritation as adverse reactions to TTO tend to fade as its concentration diminishes which unintentionally inverts the proportion in favour of Terpinen- 4-ol (Pazyar, Yaghoobi, Bagherani and Kazerouni, 2012). A study by Lee et al, (2013) identified that whilst Terpinen-4-ol exhibits strong antimicrobial and anti-inflammatory properties, the minor components do contribute to the overall antimicrobial effects of TTO.

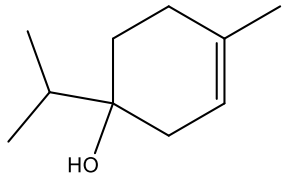

Figure 2 | Terpinen-4-ol

Whilst Lee et al (2013) investigated the antimicrobial effects of the components of TTO, clinical research into the effect of TTO on acne has been performed by numerous researchers. Bassett, Barnetson and Pannowitz, (1990) compared 5% TTO gel with 5% Benzoyl peroxide (BPO) in the treatment of mild to moderate acne in a single-blind trial. It was found that both TTO and BPO has a significant effect in reducing the number of inflammatory and non-inflammatory lesions. However, the onset of action was slower in TTO but fewer side effects were experienced. Further research by Enshaieh, Jooya, Siadat and Iraji (2007) compared 5% TTO to a placebo in a double-blind placebo-controlled study. TTO was found to reduce both inflammatory and non-inflammatory lesions in patients with mild to moderate acne. TTO was proven to be 3.55 times and 5.75 times more effective in reducing total lesion counts (TLC) and acne severity index scores (ASI) in comparison to the placebo. This study further clarified the work by Bassett, Barneston and Pannowitz (1990) in confirming the effectiveness of TTO as an acne treatment.

Most EO are used in blends or synergisms with the intention to create an effect where the combination is more powerful than the individual product (Orchard and van Vuuren, 2017); this information alongside earlier research provided a basis for investigations of TTO alongside other natural products. Mazzarello et al, (2018) produced two double-blind investigations surrounding the effect of a product containing 20% propolis, 3% TTO and 10% Aloe vera (PTAC) as they had proven antibacterial and anti-inflammatory properties. This was compared to 3% erythromycin cream. Results indicated that although PTAC did not possess sebum-reducing properties it did improve the erythema index of papules and erythematous scarring greater than the erythromycin cream. In fact, PTAC reduced the ASI score by 68% and TLS by 64% after 30 days usage in comparison to the 50% and 47% retrospectively for erythromycin cream. This study proves that natural products can be considered as an alternative treatment method to antibiotics.

The abundance of research on the use of TTO seems to indicate that it would be suitable and beneficial as an acne treatment; with TTO synergism with other natural products as an additional area of investigation. However, more clinically controlled trials need to be completed for it to be taken seriously as a medical alternative to antibiotics. In the meantime, perhaps the popularity of TTO over the counter products can bridge this gap.

Green Tea extracts

Despite it not being an EO, Camellia sinensis (L.) Kuntze (Theaceae) -commonly known as Green Tea (GT)- is another natural product that has gained interest in recent years due to its composition of polyphenols which are found in abundance in a variety of foods such as nuts, wine, vegetables and various teas which gives rise to anti-inflammatory and antimicrobial properties (Fox et al, 2016). Tea is the second most consumed beverage worldwide and therefore an important source of plant polyphenols so it is understandable that the antimicrobial properties of GT would be of interest in acne treatment (Saric, Notay and Sivamani, 2016). GT polyphenols are primarily composed of catechins of which there are several that make up the 30-42% of the extracted solid weight percentage; with the remainder being composed of flavonols (Saric, Notay and Sivamani, 2016). The most abundant catechin in GT is that of epigallocatechin-3-gallate (EGCG) (Figure 3)

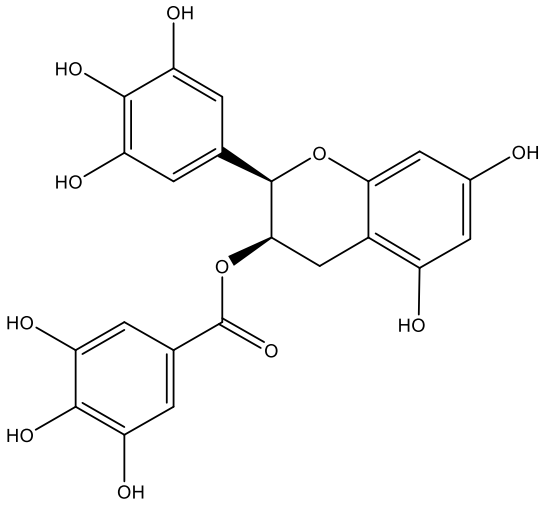

Figure 3 | (−)-Epigallocatechin gallate

Investigation into EGCG and GT has progressed over the years moving from in vitro to clinical studies with a recent study involving the ingestion of GT capsules to investigate its effects on acne (Yoon et al, 2013, Mahmood, 2013; Lu and Hsu, 2016). One of the major contributors into the effect of this natural product is by Yoon et al, (2013) who initially performed an in vitro study analysing the effects of EGCG and identified that this compound has the ability to act on three of the pathological processes of acne pathogenesis; it can suppress sebum production, inhibit the growth of *C. acnes* whilst promoting anti-inflammatory effects. Therefore, Yoon et al, moved to a double-blind split body face trial to investigate the effects of a 1% and 5% EGCG topical in comparison to a 3% ethanol topical. Each group (1% or 5% EGCG) applied the topical to one side of their face and the ethanol vehicle to the other. After eight weeks, the patients were assessed and non-inflammatory lesions were reduced alongside inflammatory lesions by 79% and 89% retrospectively for the 1% ECGC group; the 5% group showed parallel improvement.

The work by Yoon et al, (2013) was further clarified in a study by Mahmood et al, (2013) who investigated the effects of a 5% GT topical and 2.5% GT in combination with a 2.5% lotus extract (GTL) topical on facial sebum production. Results from this study shows that the group receiving the GT topical displayed a 27% reduction in sebum production from baseline where those that applied the GTL topical showed a 25% reduction. Although those receiving the GTL showed a smaller percentage reduction, the sebum secretion reduction was to a higher degree. However, a study by Lu and Hsu, (2016) investigated whether green tea supplementation could improve acne. They used 1500mg decaffeinated green tea daily (three 500mg capsules after meals) in comparison to a cellulose control; after four weeks there was no significant difference in lesion counts. This study identifies the need for topical therapies in acne.

Despite the lack of results from Lu and Hsu (2016) regarding GT supplements, there is still ample research to favour the use of GT as an acne treatment, especially given the results of GT and lotus synergism. Further research into GT synergism could be a productive move forward.

CONCLUSION

The treatment of acne is an important area of research and is gaining momentum to further understand the pathogenesis of the condition and any new potential target areas. Whilst it is known that *C. acnes* is a major contributor to the development of acne lesions, targeting of the inflammatory pathways that are initiated as a result of *C. acnes* seems a natural response. Whilst it is apparent that acne is not a typical bacterial infection but treatment with antibiotics alleviates the symptoms somewhat; consideration needs to be made as to whether this is the correct course of action with multi-drug resistant organisms on the rise and the ability of *C. acnes* strains to produce resistance relatively quickly. With CAM therapies now an area of interest specifically EO due to their complexity how that translates into multiple mechanisms of action, there is a suggestion that resistance to these natural products is less likely to develop, with no significant antimicrobial resistance reported thus far. With the copious research regarding the antimicrobial and anti-inflammatory properties of EO evident it seems obvious that they would make a suitable alternative to antibiotics. Withholding the use of antibiotics in acne has already been seen to translate into lower resistance levels in some countries; accepting the same response in every country could result in the levels of resistance being maintained if not lowered globally.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Agnew, T., M.L., Segal, L. 2014. The Clinical Impact and Cost-Effectiveness of Essential Oils and Aromatherapy for the Treatment of Acne Vulgaris: A Protocol for a Randomized Controlled Trial. The Journal of Alternative and Complementary Medicine, 20, 399-405.

Bassett, I., Barnetson, R. and Pannowitz, D., 1990. A comparative study of tea-tree oil versus benzoylperoxide in the treatment of acne. Medical Journal of Australia, 153(8),455-458.

Betts J.G., Young K.A., Wise J.A., Johnson E., Poe B., Kruse D.H., Korol O., Johnson J.E., Womble M., DeSaix P.. 2013Anatomy and Physiology. OpenStax. Houston, Texas.Book URL: https://openstax.org/books/anatomy-and-physiology/pages/1-introduction Section URL: https://openstax.org/books/anatomy-and-physiology/pages/5-4-diseases-disorders-and-injuries-of-the-integumentary-system

Christensen, G. and Brüggemann, H. (2014). Bacterial skin commensals and their role as host guardians. Beneficial Microbes, 5(2), 201-215.

Brzuszkiewicz, E., Lomholt, H. and Brüggemann, H. 2016. Antagonism between Staphylococcus epidermidis and Propionibacterium acnes and its genomic basis. BMC Genomics, 17(152), pp.1-14.

Crawford, W., Crawford, I., Stoughton, R. and Cornell, R., 1979. Laboratory Induction and Clinical Occurrences of Combined Clindamycin and Erythromycin Resistance in Corynebacterium acnes. Journal of Investigative Dermatology, 72(4),187-190.

Department of Health., 2016. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Presented to the UK Prime Minister [online] London: Department of Health. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf [Accessed 21st October 2019]

Dessinioti, C., Katsambas, A., 2017. Propionibacterium acnes and antimicrobial resistance in acne. Clinics in Dermatology, 35, 163-167.

Dréno, B. (2017). What is new in the pathophysiology of acne, an overview. Journal of the European Academy of Dermatology and Venereology, 31(5),8-12.

Dréno, B., Pécastaings, S., Corvec, S., Veraldi, S., Khammari, A. and Roques, C., 2018. Cutibacterium acnes (Propionibacterium acnes) and acne vulgaris: a brief look at the latest updates. Journal of the European Academy of Dermatology and Venereology, 32(2),5-14.

Elshafie, H. and Camele, I., 2017. An Overview of the Biological Effects of Some Mediterranean Essential Oils on Human Health. BioMed Research International, 2,1-14.

Enshaieh, S., Jooya, A., Siadat, A. and Iraji, F., 2007. The efficacy of 5% topical tea tree oil gel in mild to moderate acne vulgaris: A randomized, double-blind placebo-controlled study. Indian Journal of Dermatology, Venereology and Leprology, 73(1),22.
Esmael, A., Hassan, M., Amer, M., Abdelrahman, S., Hamed, A., Abd-raboh, H. and Foda, M., 2019. Antimicrobial activity of certain natural-based plant oils against the antibiotic-resistant acne bacteria. Saudi Journal of Biological Sciences, 27(1),448-455.
Farrah, G., Tan, E., 2016. The use of oral antibiotics in treating acne vulgaris: a new approach. Dermatologic Therapy, 29, 377-384.
Fitz-Gibbon, S., Tomida, S., Chiu, B., Nguyen, L., Du, C., Liu, M., Elashoff, D., Erfe, M., Loncaric, A., Kim, J., Modlin, R., Miller, J., Sodergren, E., Craft, N., Weinstock, G. and Li, H., 2013. Propionibacterium acnes Strain Populations in the Human Skin Microbiome Associated with Acne. Journal of Investigative Dermatology, 133(9),2152-2160.
Fox, L., Csongradi, C., Aucamp, M., du Plessis, J. and Gerber, M., 2016. Treatment Modalities for Acne. Molecules, 21(1063),1-20.
Greener, M., 2016. Managing acne: The importance of considering resistance. British Journal of School Nursing, 11(7),332-335.
Grice, E. and Segre, J., 2011. The skin microbiome. Nature Reviews Microbiology, 9(4), pp.244-253.
Hall, C. and Mah, T., 2017. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiology Reviews, 41(3),276-301.
Humphrey, S., 2012. Antibiotic Resistance in Acne Treatment. Skin Therapy Letter, 17(9),1-7.
Jahns, A., Lundskog, B., Ganceviciene, R., Palmer, R., Golovleva, I., Zouboulis, C., McDowell, A., Patrick, S. and Alexeyev, O., 2012. An increased incidence of Propionibacterium acnes biofilms in acne vulgaris: a case-control study. British Journal of Dermatology, 167(1),50-58.
Kwon, H., Yoon, J., Park, S. and Suh, D., 2013. Analysis of distribution patterns of Propionibacterium acnes phylotypes and Peptostreptococcus species from acne lesions. British Journal of Dermatology, 169(5),1152-1155.
Lee, C., Chen, L., Chen, L., Chang, T., Huang, C., Huang, M. and Wang, C., 2013. Correlations of the components of tea tree oil with its antibacterial effects and skin irritation. Journal of Food and Drug Analysis, 21,169-176.
Leyden, J.J.,1976. Antibiotic resistance acne. Cutis, 17, 593-596.
Lu, P. and Hsu, C., 2016. Does supplementation with green tea extract improve acne in post-adolescent women? A randomized, double-blind, and placebo-controlled clinical trial. Complementary Therapies in Medicine, 25,159-163.
Mahmood, T., Akhtar, N. and Moldovan, C., 2013. A comparison of the effects of topical green tea and lotus on facial sebum control in healthy humans. Hippokratia, 17(1),64-67.
Mazzarello, V., Donadu, M., Ferrari, M., Piga, G., Usai, D., Zanetti, S. and Sotgiu, M., 2018. Treatment of acne with a combination of propolis, tea tree oil, and Aloe vera compared to erythromycin cream: two double-blind investigations. Clinical Pharmacology: Advances and Applications, 10,175-181.
O'Neill, A. and Gallo, R., 2018. Host-microbiome interactions and recent progress into understanding the biology of acne vulgaris. Microbiome, 6(177),1-16.
Orchard, A. and van Vuuren, S., 2017. Commercial Essential Oils as Potential Antimicrobials to Treat Skin Diseases. Evidence-Based Complementary and Alternative Medicine, 2017,1-92.
Owen, O., M.G., Aroo, R., Ruiz-Rodado, V., Price, P., Laird, K. 2017. A Multifactorial Comparison of Ternary Combinations of Essential Oils in Topical Preperations to Current Antibiotic Prescription Therapies for the Control of Acne Vulgaris-Associated Bacteria. Phytotherapy Research, 31, 410-417.
Paugam, C., Corvec, S., Saint-Jean, M., Le Moigne, M., Khammari, A., Boisrobert, A., Nguyen, J., Gaultier, A. and Dréno, B., 2017. Propionibacterium acnes phylotypes and acne severity: an observational prospective study. Journal of the European Academy of Dermatology and Venereology, 31(9), 398-399.
Pazyar, N., Yaghoobi, R., Bagherani, N. and Kazerouni, A., 2012. A review of applications of tea tree oil in dermatology. International Journal of Dermatology, 52(7),784-790.
Plant, R., Dinh, L., Argo, S. and Shah, M., 2019. The Essentials of Essential Oils. Advances in Pediatrics, 66(2019), 111-122.
Platsidaki, E. and Dessinioti, C. (2018). Recent advances in understanding Propionibacterium acnes (Cutibacterium acnes) in acne. F1000Research, 7, 1953.

Resch, A., Rosenstein, R., Nerz, C. and Gotz, F., 2005. Differential Gene Expression Profiling of Staphylococcus aureus Cultivated under Biofilm and Planktonic Conditions. Applied and Environmental Microbiology, 71(5), 2663-2676.
Rocha, M. and Bagatin, E., 2017. Skin barrier and microbiome in acne. Archives of Dermatological Research, 310,181-185.
Ross, J., Snelling, A., Carnegie, E., Coates, P., Cunliffe, W., Bettoli, V., Tosti, G., Katsambas, A., Galvan Perez Del Pulgar, J., Rollman, O., TOrOk, L., Eady, E. and Cove, J., 2003. Antibiotic-resistant acne: lessons from Europe. British Journal of Dermatology, 148(3),467-478.
Salem, I., Ramser, A., Isham, N. and Ghannoum, M., 2018. The Gut Microbiome as a Major Regulator of the Gut-Skin Axis. Frontiers in Microbiology, 9,1-14.
Sardana, K. and Garg, V., 2014. Antibiotic resistance in acne: is it time to look beyond antibiotics and Propionobacterium acnes? International Journal of Dermatology, 53(7), 917-919.
Saric, S., Notay, M. and Sivamani, R., 2016. Green Tea and Other Tea Polyphenols: Effects on Sebum Production and Acne Vulgaris. Antioxidants, 6,2.
Schommer, N. and Gallo, R., 2013. Structure and function of the human skin microbiome. Trends in Microbiology, 21(12),660-668.
Tariq, S., Wani, S., Rasool, W., Shafi, K., Bhat, M., Prabhakar, A., Shalla, A. and Rather, M., 2019. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. Microbial Pathogenesis, 134(2019),103580.
Thursby, E. and Juge, N., 2017. Introduction to the human gut microbiota. Biochemical Journal, 474(11), pp.1823-1836. Treatment Modalities for Acne. Molecules, 21, 1-20.
Walsh, T.R., Efthimiou, J., Dreno, B. 2016. Systematic review of antibiotic resistance in acne: an increasing topical and oral threat. The Lancet, 16, 22-32.
Whitehouse, H., Fryatt, E., El-Mansori, I., Layton, A.M., 2016. Oral antibiotics for acne: are we adopting premium use? British Journal of Dermatology, 175, 91
Winkelman, W., 2018. Aromatherapy, botanicals, and essential oils in acne. Clinics in Dermatology, 36,299-305.
Wińska, K., Mączka, W., Łyczko, J., Grabarczyk, M., Czubaszek, A. and Szumny, A., 2019. Essential Oils as Antimicrobial Agents—Myth or Real Alternative? Molecules, 24, 2130.
Xu, H., Li, H. 2019. Acne, the Skin, and Antibiotic Treatment American Journal of Clinical Dermatology, 20, 335-344.
Yoon, J., Kwon, H., Min, S., Thiboutot, D. and Suh, D., 2013. Epigallocatechin-3-Gallate Improves Acne in Humans by Modulating Intracellular Molecular Targets and Inhibiting P. acnes. Journal of Investigative Dermatology, 133(2),429-440.

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Review

THE USE OF NATURAL PRODUCTS IN 3D PRINTING OF PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Background

Three-dimensional printing (3DP) has been investigated widely for applications in pharmaceutical sciences. Different 3DP techniques have been employed such as fused deposition modelling (FDM 3DP), powder bed 3DP, stereolithography 3DP (SLA 3DP), selective laser sintering (SLS 3DP), pates-extrusion 3DP and inkjet 3DP.

Aim

This article aims to explore the use of natural products as active ingredient or excipient.

Methods

Literature search was conduced for latest applications of 3DP for pharmaceutical dosage forms, and typical employed materials were identified.

Results

Polymeric materials form the main bulk of 3DP excipients such as polyvinyl alcohol or ploy lactic acid. Chemical stabilisers may be added to these polymers to increase their stability at high temperatures during hot melt extrusion for making filaments or printing. In addition, photoinitiators have been added such as diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide in SLA 3DP, or candurin gold sheen in SLS 3DP. Presence of lead has been detected in FDM £DP, which originated from the nozzle. Currently, natural products have been employed only in paste extrusion 3DP of pharmaceutical dosage forms. We have identified a protentional natural thermoplastic polymer that may be used in 3DP FDM.

Conclusion

Natural products may be employed in 3DP of pharmaceutical dosage forms to improve the safety profile of printed objects.

Keywords: Three-dimensional printing; polymers; thermoplastics; photoinitiators, natural polymers.

INTRODUCTION

3D printing (3DP) of pharmaceutical dosage forms is expanding fast.(Melocchi et al, 2020; Prasad & Smyth, 2016) This technique provides a variety of dosage forms that cannot be prepared by conventional methods, as well as the opportunity for preparing personalised medicine. Spritam is a 3D printed tablet that has acquired the FDA approval, setting the grounds for the utilization of 3D printing for the preparation of drug delivery systems, but in particular for oral dosage forms. Spritam tablets contain 250, 500, 750, or 1000 mg of levetiracetam for oral suspension. It is reported that each Spritam tablet contains the following inactive ingredients: colloidal silicon dioxide, glycerin, mannitol, microcrystalline cellulose, polysorbate 20, povidone, sucralose, butylated hydroxyanisole, both natural and artificial spearmint flavour (https://www.spritam.com/pdfs/spritam-full-prescribing-information.pdf). While in this formulation major polymers such as poly lactic-acid (PLA) was not employed, polymers play a major role in the formulation of 3DP pharmaceuticals. There is a high demand for PLA, as it is a biobased polymer derived from biomass, that degrades in the environment or biological systems rapidly into non-toxic compounds. Lactic acid can be produced by a synthetic method or microbial fermentation.(Jem & Tan, 2020) However, polymers are not pure materials. They contain residues of stabilisers, catalyst, and initiators.(Ball et al, 2012) Depending on the route of administration, these impurities could have health issues and be harmful.(Stults et al, 2015) For example, polynuclear aromatics (found in sulfur-cured elastomers) are carcinogenic.(Norwood et al, 2008) Irgafos 168 is an antioxidant which is added to polymers,(Hermabessiere et al, 2020) and this compound can be degraded to bis(2,4-di-tert-butylphenyl) phosphate,(Dorival-García et al, 2018) which has cell toxicity.(Hammond et al, 2013) PLA is usually processed by melting and this affects significantly on the stability and mechanical properties of PLA. A process that is performed regularly in 3DP. It has been suggested to add Ifragos 168 (in combination with other antioxidant Irgnox 1076) to improve the stability of PLA during the production process involving melting of the polymer.(Oliveira et al, 2016)

These compounds are additives to polymers. The majority of such impurities are common chemical additives used to improve the physicochemical properties of a wide range of plastic materials and these appear as extractables or leachables in pharmaceutical products.(Li et al, 2015) The term extractables refers to a profile of extracted compounds found in studies under harsh conditions, but the term leachables refers to those impurities that leach from the materials under real-use conditions and may be present in final drug products. In the development of a drug product, careful consideration should be given to impurities that may originate from manufacturing equipment, process components, and packaging materials. Normally, plastics are not consumed in the drug delivery systems, however, in 3D printing these plastics may contain active ingredients and taken orally. Therefore, suppliers and drug manufacturers for 3DP should conduct studies to identify chemical additives from the plastic materials in order to screen and predict potential health issues in particular if taken on a regular basis. Clearly, biomaterials play an increasing role in contemporary intelligent drug delivery technologies as well as modern health care systems. Identifying biocompatible material poses a significant challenge for both researchers and manufacturers of modern drug delivery systems from material development to market approval.

In the following sections different 3DP techniques are introduced that have been employed for pharmaceutical dosage forms. In addition, typical materials are provided with potential health/toxicity issue. Then potential natural products are explained that can be substituted.

DIFFERENT 3DP METHODS

Fused Deposition Modelling

Fused deposition modelling (FDM) is commonly used 3DP in preparation of solid dosage forms.(Ehtezazi et al, 2018; Gorkem Buyukgoz et al, 2020; Gültekin et al, 2019; Ibrahim et al, 2019; Kempin et al, 2018; Kempin et al, 2017; Okwuosa et al, 2018; Reddy Dumpa et al, 2020; Solanki et al, 2018; Tagami et al, 2019; Wei et al, 2020) In this technique, normally a filament is inserted into the printer which melts and extrudes the filament through a narrow nozzle, typically 0.4 mm diameter, although nozzles are available with diameters as large as 1 mm for printing highly viscous compounds. The molten filament is deposited on a platform, like glass, according to the design created using the slicer software. The rastered back and forth movement of the printer head leaves the molten material side-by-side, or more interestingly the printhead is fixed but the build-plate moves in different directions (x, y, z). When one layer is complete, then the z-axis movement of the platform deposits the molten filament in the layer above. This process is

repeated until the object is fully materialised, and the molten state of the filament attaches the layers. FDM 3D printers can produce objects with uniform drug distribution,(Trenfield et al, 2018) and reproducible dimensions, in particular when filaments are used with uniform diameters (low diameter tolerance).(Goyanes et al, 2015) The drug is usually loaded during the preparation of the filament,(Ehtezazi et al, 2018) however, soaking a blank filament into the drug solution may be used.(Tagami et al, 2019) Typical materials and temperatures used in FDM 3DP are: hydroxy propyl cellulose (140-145°C),(Gorkem Buyukgoz et al, 2020) polyvinyl alcohol (180-200°C),(Ehtezazi et al, 2018; Wei et al, 2020) ethyl cellulose (165°C), (Reddy Dumpa et al, 2020) polyvinyl pyrrolidone (100°C),(Kempin et al, 2018) polyethylene glycol 20,000 (100°C),(Kempin et al, 2018) hydroxypropyl methylcellulose acetate succinate (150-170°C),(Solanki et al, 2018) polycaprolactone (47-140°C),(Kempin et al, 2017)and poly(ethylene) oxide (120-130°C).(Gültekin et al, 2019) Rindelaub et al 2019 identified extractable profiles from different grades of PLA. Surprisingly lead (Pb) was found in the printlets in the range of 0.11-1.46 ng/g, which was originated from the printing nozzle.(Rindelaub et al, 2019) In addition, Irganox 1010 (an antioxidant) was found in FDA approved PLA at the level of 1232 µg/g.(Rindelaub et al, 2019)

Filaments are produced in the temperature range of 47-200°C.(Gorkem Buyukgoz et al, 2020; Gültekin et al, 2019; Kempin et al, 2018; Kempin et al, 2017; Reddy Dumpa et al, 2020; Solanki et al, 2018; Wei et al, 2020) The temperature of the 3DP normally is higher than the filament extrusion temperature.(Kempin et al, 2017) This is because the 3DP nozzle diameter is smaller than extrusion nozzle (die) diameter; and for viscose molten polymers larger nozzle diameters are needed to allow flow of molten polymer from the nozzle, otherwise the nozzle will become blocked.

As FDM requires a filament and manufacturing of the filament may appear as a barrier, direct 3DP has been invented.(Goyanes et al, 2019a) The powder blend is added directly into the printer head. Hydroxypropyl cellulose (HPC) was employed with this printer with printing temperature of 170°C. Recently, direct 3DP was employed to produce tramadol printlets using HPC.(Ong et al, 2020) Fanous et al. 2020 employed direct 3DP to produce immediate release tablets at printing temperature of 155-180°C.(Fanous et al, 2020) PEG4000 and Kollidon VA64 were added to achieve rapid release.

Powder bed 3D printing

In powder bed 3D printing, a liquid (ink) is deposited on a flat layer of powder, and the ink causes adhesion of the solid particles together. A defined shape is formed by precise movements of the printer head. When one layer is formed, the platform is lowered for the thickness of one layer and then the old layer is covered with fresh powder, and the cycle starts again. Normally, the ink is a binder solution.(Katstra et al, 2000) Spritam is produced by this method, and Spritam is the only approved 3DP dosage form so far. However, clogging of the inkjet nozzle is the main challenge, when the ink contains a binder. Infanger et al. 2019 overcame this problem by using water and ethanol mixture as the ink, but including HPC as the solid binder.(Infanger et al, 2019) However, the 3D printed tablets disintegrated in the range of 131-1854 s depending on the type of HPC. While Spritam is known for disintegration in less than 5 s. Katstra et al. 2000 were applied powder 3D printing to formulated porous tablets; and a solution of Eudragit E100 in ethanol (20% w/w) was used as the binder solution. The powder bed was Avicel PH301.(Katstra et al, 2000) While Wu et al 1996, prepared a solution of poly-ε-caprolactone (PCL) in chloroform (5% w/w) to be deposited on polyethylene oxide (PEO) and PCL powder.(Wu et al, 1996) An inkjet nozzle with 45 µm was utilised. This interesting drug delivery design had walls of PCL (with different thicknesses) with internal compartments made of PEO. Yu etal 2009 employed powder bed 3DP to produce drug delivery devices that provided linear drug release profiles.(Yu et al, 2009) The binder solution contained ethyl cellulose in ethanol, or active ingredient (paracetamol) in ethanol.

The infiltration of the binder solution (ink) through the porous structure of powder bed is governed by capillary forces, and this infiltration determines the printing resolution. Barui et al. 2020 demonstrated that the ink (ethylene glycol) could penetrate within one second to the depth of a model powder (alumina).(Barui et al, 2020)

Stereolithography (SLA) 3D printing

In SLA 3D printing laser light is employed. In this method a resin such as polyethylene glycol diacrylate (PEGDA) is held inside a container with a photoinitiator (PI) such as diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (DPPO).(Wang et al, 2016) Then precise movement of laser light (such

as HeCd) in a 2D pattern, cures the resin, also known as photopolymerisation. When a layer is cured, then the platform with the cured structure attached is lowered in the bottom-up approach and therefore, another layer of uncured liquid resin spreads over the top. The SLA provides the opportunity of 3DP by bottom-to-top approach, where the laser light is emitted from the bottom of the resin pool. Usually, the drug molecule is dissolved in the resin solution. The resolution of SLA 3DP is much higher than FDM;(Kjar & Huang, 2019) and it is in the range of 30-140 µm, or as small as 16 µm.(Chia & Wu, 2015) In the SLA 3DP, the kinetics of the curing reaction are critical, which depend on energy imparted by the laser and the power of the light source, the scanning speed, the exposure time and the amount of polymer and photoinitiator.(Chia & Wu, 2015) Wang et al. 2016, applied SLA to produce oral dosage forms loaded with 4-aminosalicylic acid (4-ASA) and paracetamol. The release of active ingredients was modified by adjusting the amounts of PEGDA.(Wang et al, 2016) The active ingredients were released from the oral dosage forms due to the dissolution of the polymer in the testing media. In another study, Martinez et. al. 2017 applied SLA 3D to produce hydrogels of ibuprofen.(Martinez et al, 2017) PEGDA was used as the photopolymerisable monomer with PEG300 and water to adjust the crosslinking density of the hydrogel. This study used two different PIs: DPPO and riboflavin (with triethanolamine as a co-initiator). The objects were printed at the resolution of 300 µm. Dissolution data showed that increasing water content increased drug release rate.(Martinez et al, 2017) Xu et al. 2020 employed SLA 3DP to produce polyprintlets (a multilayer polypill).(Xu et al, 2020) To achieve this, the printer software was modified, which allowed changing the resin solution containing different active ingredients. Undesirable reactions between API and photoreactive monomers should be investigated or eliminated when SLA 3DP is employed.

In Process Drying 3DP

In process drying 3DP is a semisolid extrusion 3DP. In this method, an aqueous based semisolid formulation was prepared that contained hydroxyethylcelulose, drug, and sorbitol as plasticizer. The 3D printer was a modified FDM that the printhead was connected to a syringe pump and a 2 mm glass sheet was used as the print surface.(Elbl et al, 2020) The printer platform was heated to 75°C to dry the printed objects. After printing, films were kept on heated bed for 10 min to ensure desired drying degree of all films. Apply in-process drying 3D printer allowed to manufacture FDFs with thickness from 45-205µm with disintegration time of less than 40 s (for 100 µm thickness films).(Elbl et al, 2020) Films with the thickness of 40 µm disintegrated within 10 s. Printing multilayer objects seems challenging with the in drying process, as initial layers dry and subsequent layers may not deposit suitably. In another approach, Sjöholm and Sandler 2019 employed semi-solid extrusion 3DP to manufacture warfarin FDFs. The films were printed on the platform of a Biobot 3DP and were left to dry over 24 hr. Interestingly, blank films (drug unloaded) disintegrated within 3 s, while drug loaded films disintegrated at much longer time.(Sjöholm & Sandler, 2019) It should be noted that the disintegration time was measured by a drop method, which is putting one film in a texture analyser film support rig and one drop of 0.2 mL purified water is placed in the hole on top of film. The time is recorded when the film breaks (the drop falls-through).(Sjöholm & Sandler, 2019)

Direct 3DP

As mentioned in the previous section, conventional FDMs require a filament to produce the 3D objects and manufacturing a suitable filament can be challenging that can withstand the mechanical stresses of printer head.(Ilyés et al, 2019) Therefore, avoiding preparation of filaments could help the development 3D printed pharmaceutical dosage forms. To achieve this, a direct powder 3DP was developed, which was based on mounting a single-screw HME on the top of the printer head. Then, the rotation speed and the extrusion were controlled by the 3DP software. The HME extruder and printer head assembly (including a nozzle with 0.8 mm diameter) moved in 3 dimensions to make the objects in a layer-by-layer style.(Goyanes et al, 2019a) Use of direct powder/pellet FDM 3DP now is becoming wider.(Liu et al, 2019) It was found that the stepper motor's speed had the most significant effect on the diameter of the printed fibres.(Liu et al, 2019) In another approach, a pneumatic direct FDM 3DP was developed, in which powder is fed into a cylinder and the content is melted using an heating element and extruded through a nozzle with 0.4 mm aperture with the help of air pressure.(Cho et al, 2020) Using this printer allowed to prepare orodispersible films that disintegrated in the range of 17-21 s. PEO and PVPVA (Kollidon® VA64) and poloxamer 188 were used in the orodispersible formulation with disintegration time of 17 s.(Cho et al, 2020)

The direct powder 3DP was employed to produce itraconazole printlets using four different grades of HPC.(Goyanes et al, 2019a) In addition, direct 3DP was employed to fabricate rapid release dosage forms

by incorporating a honeycomb structure into the printlets.(Fanous et al, 2020) Furthermore, direct powder 3DP was used to prepare nifedipine minitablets containing 20 mg of API with 15% PEG 4000 Da, 40% HPC, 19% hydroxy propyl methyl cellulose acetate succinate, and 1% magnesium stearate.(Sánchez-Guirales et al, 2021) An Engine SR Hyrel FDM 3DP was used to print the minitablets, but a modular head TAM-15® extruder was used to extrude the powder through a 1 mm aperture nozzle. M3DIMAKER™ pharmaceutical direct powder 3D printer was employed to produce paediatric praziquantel printlets.(Boniatti et al, 2021) As praziquantel has an unpleasant taste, then splitting conventional tablets exposes the taste to paediatric patients and compromising their compliances. Therefore, 3DP allowed to produce personalised tablets without the need for adjusting the dose by splitting a larger dose tablet.

Direct 3DP has its own challenges. Usually, direct 3DP nozzles are greater than conventional 3DP FDM, therefore, fine resolutions may not be achievable. Furthermore, the viscosity of molten powder could be high and therefore, stronger electromotors are needed. However, these are bulky, and their torque may damage the printhead (authors experience with initial Noztek extruder that with certain powders the viscosity was so high that the electromotor of the extruder twisted the chassis of the extruder and the whole frame got damaged. As a result, new Noztek extruders have a sensor that above a certain force the extruder shuts off). Therefore, these could be the reasons why direct 3DP has not been used extensively in the formulation of pharmaceutical dosage forms.(Fina et al, 2020) Furthermore, the cleaning of the head can be difficult due to small spaces and using brushes. Therefore, direct 3DP can be useful only for a small range of material. Advanced extruders have three different temperature regions with twin screws, while achieving these arrangements in a 3D printhead may be challenging.

Two-Photon Polymerisation 3DP

In two-photon lithography (TPL) 3DP, a liquid material is converted into solid by light.(Harinarayana & Shin, 2021) The liquid contains monomer and by absorption of two photons (two laser beams) in the infrared range polymerisation is initiated and small voxel of the liquid is solidified. The laser emitting duration is short in the range of 100 femtosecond. This leads to a short photopolymerisation, therefore basically, it is pin-pointing polymerisation. This techniques allows to fabricate at sub-micro resolution (0.5 µm, i.e. a castle model can be built on the tip of a pencil).(Maibohm et al, 2020) A suitable material for TPL-3DP has two components: 1) a monomer or mixture of monomers, 2) photoinitiator.(Selimis et al, 2015) SU8 is one of the widely used material in TPL.(Selimis et al, 2015) Both monomer and photoinitiator must be transparent at the laser wavelength, so the laser beam can penetrate inside the liquid, and not being absorbed at the surface.(Selimis et al, 2015) Preloading of drug molecules have been suggested into fine structures that are prepared by TPL-3DP.(Limongi et al, 2020) In another approach, TPL-3DP was employed to manufacture templates of microneedle arrays for transdermal drug delivery.(Cordeiro et al, 2020) The ultra-low resolution of the TPL-3DP allowed to produce templates with needle length in the range of 900-1300 µm with different shapes (pyramidal, conical). These templates permitted to produce dissolving microneedle arrays made from PVP and PVA.(Cordeiro et al, 2020) Do et al 2018 applied TLP-3DP to produce drug delivery devices made of PEG dimethacrylate (PEGDMA). Irgacure 369 was employed as the photoinitiator. The model drug was dissolved in the PEGDMA and photoinitiator aqueous solution.(Do et al, 2018) Devices were manufactured with pore sizes in the range of 5-15 µm, which allowed to control drug release from the devices. The devices were found biocompatible by presenting no cell viability issues. In fact, the toxicological aspects of fabrication process would be the point of concern due to presence of free radicals following polymerisation.

Hot Melt Ram and Hot-Melt Pneumatic Extrusion 3DP

Other approaches to avoid filament manufacturing are hot-melt ram and hot-melt pneumatic extrusion 3DP. In hot-melt ram 3DP, maltodextrins, drug, and other excipients were mixed (in a mortar) and coated with a plasticizer (i.e. glycerine). Then, the mixture was fed in the cylinder (chamber) of the ram-extruder, which was connected to an 18G needle. The cylinder was heated to melt the mixture. It should be noted that the whole cylinder down to the needle was covered by a thermostated support. This is essential to ensure that mixture stays molten within the 3DP. The ram-extruder was mounted on a 3DP FDM, to achieve a raster fashion movement. This method was employed to manufacture orodispersible films, which disintegrated in less than 1 min (73 ± 15s).(Musazzi et al, 2018) The hot-melt pneumatic extrusion 3DP is similar to hot-melt ram extrusion 3DP, but instead of ram, the molten mixture was extruded with applying air pressure. Oh et. al. 2020 applied hot-melt pneumatic extrusion 3DP to produce orodispersible films by employing

PEO (100k Da), ploxamer 188 and citric acid. The films disintegrated in less than 25 s, (Oh et al, 2020) and the air pressure was in the range of 250-350 kPa. Furthermore, hot-melt pneumatic extrusion was employed to produce 3DP tablets of dutasteride.(Kim et al, 2021) Th excipients were: Soluplus®, Kollidon® VA 64, Eudragit® E PO, and HPC. The nozzle diameter was 0.4 mm, indicating of printlets with a high resolution. However, the tablets were printed at 160°C-190°C, which means that a large quantity of the formulation was kept at high temperatures, potentially causing drug stability issue. (Kim et al, 2021)

Paste-Extrusion 3DP

In paste-extrusion 3DP, a gel-based material (semisolid) is filled inside a syringe, which the feedstock is connected to a stepper motor, (Amza et al, 2017) or air pressure(Tagami et al, 2021) to pass the paste through an extrusion nozzle. Paste-extrusion 3DP has been employed to produce gummies that contain active ingredients.(Goyanes et al, 2019c; Herrada-Manchón et al, 2020; Rycerz et al, 2019; Tagami et al, 2021) Herrada-Manchón et al 2020 developed a paste formulation comprising gelatine, corn starch, carrageenan, and xanthan gum with ranitidine as the active ingredient.(Herrada-Manchón et al, 2020) The gel was heated up to 37°C and then was filled into 3 ml syringes followed by 3D printing. The printhead temperature was set to 37°C too, with printing bed at 15°C, which help rapid solidifying of the printed gummy. Printlets with suitable finish were obtained with 80% infill density. Solidification of the gel in the syringe is one of the drawbacks of this approach. Therefore, Tagami et al 2021 developed a gel formulation containing gelatine, HPMC, and reduced syrup with lamotrigine as the active ingredient.(Tagami et al, 2021) This formulation was not set at room temperature and extruded through a nozzle (27 G, 0.413 mm internal diameter). However, the printed objected were dried overnight at room temperature. This formulation led to printing main shapes such as star or disk, while it appears printing object with the shape of animals could be challenging. Meaning that if gels are solidified at higher temperatures, then there is a better chance of printing with fine details. It should be noted that we found the paste formulation with HPMC could solidify at temperatures less than 25°C (when the environment temperature is cold such autumn or winter). Therefore, we are developing a paste extruder with heating jacket and the results will be published shortly. The pate-extrusion 3DP has been tested in paediatric patients (3-16 year) for the delivery of isoleucine.(Goyanes et al, 2019b) The chewable printlets were only in the shape of cylinders, however, they were accepted by the patients. The above information indicates that the paste-extrusion 3DP may employ natural products to FDM 3DP or SLA 3DP. Table 1 presents the use/potential use of natural products in 3DP of pharmaceutical dosage forms.

Selective Laser Sintering 3DP

Selective laser sintering 3DP (SLS 3DP) involves heating powder particles by laser leading to melt and fusion of powder particles.(Charoo et al, 2020a) As it would be expected the resolution of SLS 3DP depends on the laser diameter in the range of 0.3 mm(Berry et al, 1997b) to 0.02 mm (20μm).(Muzaffar et al, 2020) As a result SLS produced an impressive 1.79% dimensional error.(Ibrahim et al, 2009) It should be noted that a laser absorbing material such as Candurin gold sheen (a food ingredient) may be required to be added to the powder mixer.(Charoo et al, 2020b) This process includes formation of a powder layer on a powder bed, where a controlled scanning laser beam fuses powder particles and a 2D layer is formed. Then another powder layer is spread over the previous layer with the help of a roller and the laser beam builds another layer over the previous built. This process is iterated until the desired object is formed, which located within a bulk of powder mass. The object is recovered and cleaned from the residual powder. Nylon is the most common material for SLS 3DP.(Berry et al, 1997a) Other potential polymers are polyethylene both high and low density, polyvinyl chloride and polystyrene.(Asim et al, 2017) Generally, thermoplastic polymers should be suitable for SLS 3DP. A thermoplastic polymer can be melt and shaped at a specific temperature.(Asim et al, 2017) However, the powders used for pharmaceutical applications are: HPMC (grades 100 to 30),(Fina et al, 2018) Kollidon® VA 64 (grades 100 to 300)(Fina et al, 2018), Kollicoat IR,(Fina et al, 2017) Eudragit L100-55(Fina et al, 2017), poly (L-lactic acid),(Duan et al, 2010) polyethylene,(Salmoria et al, 2018) poly (lactic-acid),(Bai et al, 2017) polyetheretherketone (PEEK),(Tan et al, 2003) and polycaprolactone.(Leong et al, 2007) SLS 3DP has been applied to produce orally disintegrating tablets (printlets),(Fina et al, 2018) with outstanding disintegration time of 4 seconds when Kollidon® VA 64 grade 300 was employed.(Fina et al, 2018) Candurin gold sheen was also added to the

Table 1. Natural products that have been used in 3DP including 3DP of pharmaceutical dosage forms.

Compound	Origin	Dosage Form/shape	3DP	Remarks	Ref
Xanthan gum	*Xanthomonas campestris*	gummy	Paste extrusion 3DP	Eye catching objects were produced	(Herrada-Manchón et al, 2020)
Pectin	Fruits such as apple and carrot	Chewable tablet	Paste extrusion 3DP	Tablets were well accepted by paediatric patients	(Goyanes et al, 2019c)
Sodium alginate	Phaeophyceae	Multilayer mesh structure	Paste extrusion 3DP	Flexible structure were prepared and CaCl2 was used to cross link alginate	(Wang et al, 2021)
Chitosan	*Litopenaeus vannamei* Boone (de Queiroz Antonino et al, 2017)	Star/half moon	Direct ink writing	Shapes with high resolutions were produced	(Zhou et al, 2020)
Snakegourd root/Astragalus	*Trichosanthes anguina* L/Astragalus propinquus	Square, round, rectangle	Hot melt extrusion 3DP	Changing shape led to change in the drug release rate	(Yan et al, 2019)
sodium hyaluronate	bovine vitreous humor/ *Streptococcus equi/*	Composite scaffolds	Paste extrusion 3DP followed by layer-by-layer coating with sodium hyaluronate	Sodium hyaluronate reduced drug release from the scaffolds	(Chen et al, 2019)
Collagen	Bone/skin/connective tissue of animals (cattle, fish, horse)	Composite scaffolds	FDM 3DP using PLA followed by coating with collagen	Scaffolds provided a closer structural support approximation to native bone architecture	(Martin et al, 2019)
Chocolate	*Theobroma cacao*	Cartoon characters	Paste extrusion 3DP	80% of drug released within 30 minutes	(Karavasili et al, 2020)

formulation. This printlet produced the least breaking force (13 N) with the highest porosity (40%).(Fina et al, 2018) Applying x-ray crystallography found that paracetamol drug crystals could be identified in 3D printed tablets made by the SLS method. While DSC analysis failed to detect the drug crystals.(Fina et al, 2017) This would be expected as by melting the thermoplastic polymer, the drug crystal may not melt at the same time and molten plastic may encapsulate the drug crystal. Intrauterine devices were manufactured using SLS 3DP. It was found that laser power had little effects on the release of progesterone or 5-

Fluorouracil.(Salmoria et al, 2018) The potential drawback of SLS 3DP is drug degradation during melting the polymers.(Fina et al, 2017) Furthermore, large quantities of powders are required and this may become costly for expensive drugs.(Awad et al, 2020)

Inkjet 3P

As explained in the previous section, inkjet 3DP has been employed for powder bed 3DP. Other applications of inkjet printing will be reviewed in this section. It should be noted that inkjet printing deposits liquid droplets and these can form solid objects, when the droplets themselves contain nanopatilces.(Saleh et al, 2017) In this approach the nanoparticles are joined by a curing or sintering process, which leaves a solid 2D layer behind. 3D objects were created with the height of 2 mm, following printing of 1000 layers.(Saleh et al, 2017) In another approach, the ink contained quantum dots and the inkjet printing was employed to uniformly deposit the quantum dots on transdermal microneedles.(Boehm et al, 2011) Uddin et al 20.15 applied this technique to coated transdermal microneedles with three anticancer drugs (curcumin, 5 fluorouracil, cisplatin).(Uddin et al, 2015) Inkjet printing have been employed to deposit salbutamol sulphate on oral films made of potato starch.(Buanz et al, 2011) In this approach, the ink contained aqueous solution of salbutamol sulphate, as well as glycerol, which was employed to increase the viscosity of the ink and prevent salbutamol sulphate crystallisation. Inkjet 3DP has been widely used in tissue engineering, where cells are deposited uniformly on a hydrogel scaffolding layer-by-layer with simultaneous photopolymerisation of the gel.(Cui et al, 2012; Gao et al, 2015; Tamay et al, 2019) Polymers such as the Soluplus, a co-polymer of polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol has been employed to coat the microneedles.(Uddin et al, 2015) The drug and polymers were dissolved in water or ethanol. Also, aqueous solution of quantum dot nanocrystals (Qtracker® 705) were used as ink for inkjet printing on microneedles.(Boehm et al, 2012) The ink may contain viscosity increasing agents and surfactants as well as nanoparticles.(Saleh et al, 2017) For cell printing, the ink contained cells, I-2959 photoinitiator, poly(ethylene glycol) dimethacrylate and gelatin methacrylate.(Gao et al, 2015) Blocking the inkjet nozzle is the main challenge. Clogging may happen for nanoparticles due to flow-induced aggregation at the nozzle or if the suspended nanoparticles are not sufficiently stabilised and aggregations form.(Lee et al, 2012)

CONCLUSIONS

Synthetic polymeric materials form most of excipients in 3DP. Thermoplastics form majority of the polymers. In certain SLS 3DPs photoinitiators are required to commence photopolymerisation. The cytotoxicity and safety of these chemicals should be investigated in pharmaceutical applications, in particular when these materials are used on a regular basis. Natural products may appear as active ingredient in the 3DP. However, they have been used as excipients in extrusion based 3DP and inkjet printing. Extrusion based 3DP have been employed to manufacture gummies containing APIs for paediatric use. Therefore, use of natural products may become desirable as natural products do not tend to carry synthetic additives. These advantages may also encourage parents and caregivers to maintain patient compliance. Shellac is a natural thermoplastic polymer with melting point in the range of 115-120°C. Shellac is used on candies as shiny shells. Furter investigations are required for the suitability of this material in 3DP, in particular in FDM 3DP.

Conflicts of Interest

The authors declare no personal or financial conflict of interest related to this work.

Authors contribution

REFERENCES

Amza, C. G., Zapciu, A. & Popescu, D. (2017) Paste Extruder—Hardware Add-On for Desktop 3D Printers. *Technologies*, 5(3), 50.

Asim, M., Jawaid, M., Saba, N., Ramengmawii, Nasir, M. & Sultan, M. T. H. (2017) 1 - Processing of hybrid polymer composites—a review, in Thakur, V. K., Thakur, M. K. & Gupta, R. K. (eds), *Hybrid Polymer Composite Materials*Woodhead Publishing, 1-22.

Awad, A., Fina, F., Goyanes, A., Gaisford, S. & Basit, A. W. (2020) 3D printing: Principles and pharmaceutical applications of selective laser sintering. *International Journal of Pharmaceutics*, 586, 119594.

Bai, J., Goodridge, R. D., Hague, R. J. M. & Okamoto, M. (2017) Processing and characterization of a polylactic acid/nanoclay composite for laser sintering. *Polymer Composites*, 38(11), 2570-2576.
Ball, D. J., Norwood, D. L. & Nagao, L. M. (2012) Overview of Leachables and Extractables in Orally Inhaled and Nasal Drug Products, *Leachables and Extractables Handbook*, 1-19.
Barui, S., Ding, H., Wang, Z., Zhao, H., Marathe, S., Mirihanage, W., Basu, B. & Derby, B. (2020) Probing Ink-Powder Interactions during 3D Binder Jet Printing Using Time-Resolved X-ray Imaging. *ACS Appl Mater Interfaces*, 12(30), 34254-34264.
Berry, E., Brown, J. M., Connell, M., Craven, C. M., Efford, N. D., Radjenovic, A. & Smith, M. A. (1997a) Preliminary experience with medical applications of rapid prototyping by selective laser sintering. *Med Eng Phys*, 19(1), 90-6.
Berry, E., Brown, J. M., Connell, M., Craven, C. M., Efford, N. D., Radjenovic, A. & Smith, M. A. (1997b) Preliminary experience with medical applications of rapid prototyping by selective laser sintering. *Medical Engineering & Physics*, 19(1), 90-96.
Boehm, R. D., Miller, P. R., Hayes, S. L., Monteiro-Riviere, N. A. & Narayan, R. J. (2011) Modification of microneedles using inkjet printing. *AIP advances*, 1(2), 22139-22139.
Boehm, R. D., Miller, P. R., Singh, R., Shah, A., Stafslien, S., Daniels, J. & Narayan, R. J. (2012) Indirect rapid prototyping of antibacterial acid anhydride copolymer microneedles. *Biofabrication*, 4(1), 011002.
Boniatti, J., Januskaite, P., Fonseca, L. B. D., Viçosa, A. L., Amendoeira, F. C., Tuleu, C., Basit, A. W., Goyanes, A. & Ré, M. I. (2021) Direct Powder Extrusion 3D Printing of Praziquantel to Overcome Neglected Disease Formulation Challenges in Paediatric Populations. *Pharmaceutics*, 13(8).
Buanz, A. B. M., Saunders, M. H., Basit, A. W. & Gaisford, S. (2011) Preparation of Personalized-dose Salbutamol Sulphate Oral Films with Thermal Ink-Jet Printing. *Pharmaceutical Research*, 28(10), 2386.
Charoo, N. A., Barakh Ali, S. F., Mohamed, E. M., Kuttolamadom, M. A., Ozkan, T., Khan, M. A. & Rahman, Z. (2020a) Selective laser sintering 3D printing - an overview of the technology and pharmaceutical applications. *Drug Dev Ind Pharm*, 46(6), 869-877.
Charoo, N. A., Barakh Ali, S. F., Mohamed, E. M., Kuttolamadom, M. A., Ozkan, T., Khan, M. A. & Rahman, Z. (2020b) Selective laser sintering 3D printing – an overview of the technology and pharmaceutical applications. *Drug Development and Industrial Pharmacy*, 46(6), 869-877.
Chen, S., Shi, Y., Luo, Y. & Ma, J. (2019) Layer-by-layer coated porous 3D printed hydroxyapatite composite scaffolds for controlled drug delivery. *Colloids and Surfaces B: Biointerfaces*, 179, 121-127.
Chia, H. N. & Wu, B. M. (2015) Recent advances in 3D printing of biomaterials. *Journal of Biological Engineering*, 9(1), 4.
Cho, H. W., Baek, S. H., Lee, B. J. & Jin, H. E. (2020) Orodispersible Polymer Films with the Poorly Water-Soluble Drug, Olanzapine: Hot-Melt Pneumatic Extrusion for Single-Process 3D Printing. *Pharmaceutics*, 12(8).
Cordeiro, A. S., Tekko, I. A., Jomaa, M. H., Vora, L., McAlister, E., Volpe-Zanutto, F., Nethery, M., Baine, P. T., Mitchell, N., McNeill, D. W. & Donnelly, R. F. (2020) Two-Photon Polymerisation 3D Printing of Microneedle Array Templates with Versatile Designs: Application in the Development of Polymeric Drug Delivery Systems. *Pharmaceutical Research*, 37(9), 174.
Cui, X., Breitenkamp, K., Finn, M. G., Lotz, M. & D'Lima, D. D. (2012) Direct human cartilage repair using three-dimensional bioprinting technology. *Tissue engineering. Part A*, 18(11-12), 1304-1312.
de Queiroz Antonino, R. S. C. M., Lia Fook, B. R. P., de Oliveira Lima, V. A., de Farias Rached, R. Í., Lima, E. P. N., da Silva Lima, R. J., Peniche Covas, C. A. & Lia Fook, M. V. (2017) Preparation and Characterization of Chitosan Obtained from Shells of Shrimp (Litopenaeus vannamei Boone). *Marine drugs*, 15(5), 141.
Do, A.-V., Worthington, K. S., Tucker, B. A. & Salem, A. K. (2018) Controlled drug delivery from 3D printed two-photon polymerized poly(ethylene glycol) dimethacrylate devices. *International Journal of Pharmaceutics*, 552(1), 217-224.
Dorival-García, N., Carillo, S., Ta, C., Roberts, D., Comstock, K., Lofthouse, S., Ciceri, E., D'Silva, K., Kierans, G., Kaisermayer, C., Lindeberg, A. & Bones, J. (2018) Large-Scale Assessment of Extractables and Leachables in Single-Use Bags for Biomanufacturing. *Anal Chem*, 90(15), 9006-9015.
Duan, B., Wang, M., Zhou, W. Y., Cheung, W. L., Li, Z. Y. & Lu, W. W. (2010) Three-dimensional nanocomposite scaffolds fabricated via selective laser sintering for bone tissue engineering. *Acta Biomaterialia*, 6(12), 4495-4505.

Ehtezazi, T., Algellay, M., Islam, Y., Roberts, M., Dempster, N. M. & Sarker, S. D. (2018) The Application of 3D Printing in the Formulation of Multilayered Fast Dissolving Oral Films. *Journal of Pharmaceutical Sciences*, 107(4), 1076-1085.
Elbl, J., Gajdziok, J. & Kolarczyk, J. (2020) 3D printing of multilayered orodispersible films with in-process drying. *International Journal of Pharmaceutics*, 575, 118883.
Fanous, M., Gold, S., Muller, S., Hirsch, S., Ogorka, J. & Imanidis, G. (2020) Simplification of fused deposition modeling 3D-printing paradigm: Feasibility of 1-step direct powder printing for immediate release dosage form production. *International Journal of Pharmaceutics*, 578, 119124.
Fina, F., Goyanes, A., Gaisford, S. & Basit, A. W. (2017) Selective laser sintering (SLS) 3D printing of medicines. *International Journal of Pharmaceutics*, 529(1), 285-293.
Fina, F., Goyanes, A., Rowland, M., Gaisford, S. & A, W. B. (2020) 3D Printing of Tunable Zero-Order Release Printlets. *Polymers (Basel)*, 12(8).
Fina, F., Madla, C. M., Goyanes, A., Zhang, J., Gaisford, S. & Basit, A. W. (2018) Fabricating 3D printed orally disintegrating printlets using selective laser sintering. *Int J Pharm*, 541(1-2), 101-107.
Gao, G., Schilling, A. F., Hubbell, K., Yonezawa, T., Truong, D., Hong, Y., Dai, G. & Cui, X. (2015) Improved properties of bone and cartilage tissue from 3D inkjet-bioprinted human mesenchymal stem cells by simultaneous deposition and photocrosslinking in PEG-GelMA. *Biotechnol Lett*, 37(11), 2349-55.
Gorkem Buyukgoz, G., Soffer, D., Defendre, J., Pizzano, G. M. & Davé, R. N. (2020) Exploring tablet design options for tailoring drug release and dose via fused deposition modeling (FDM) 3D printing. *International Journal of Pharmaceutics*, 591, 119987.
Goyanes, A., Allahham, N., Trenfield, S. J., Stoyanov, E., Gaisford, S. & Basit, A. W. (2019a) Direct powder extrusion 3D printing: Fabrication of drug products using a novel single-step process. *International Journal of Pharmaceutics*, 567, 118471.
Goyanes, A., Buanz, A. B., Hatton, G. B., Gaisford, S. & Basit, A. W. (2015) 3D printing of modified-release aminosalicylate (4-ASA and 5-ASA) tablets. *Eur J Pharm Biopharm*, 89, 157-62.
Goyanes, A., Madla, C. M., Umerji, A., Duran Piñeiro, G., Giraldez Montero, J. M., Lamas Diaz, M. J., Gonzalez Barcia, M., Taherali, F., Sánchez-Pintos, P., Couce, M.-L., Gaisford, S. & Basit, A. W. (2019b) Automated therapy preparation of isoleucine formulations using 3D printing for the treatment of MSUD: First single-centre, prospective, crossover study in patients. *International Journal of Pharmaceutics*, 567, 118497.
Goyanes, A., Madla, C. M., Umerji, A., Duran Piñeiro, G., Giraldez Montero, J. M., Lamas Diaz, M. J., Gonzalez Barcia, M., Taherali, F., Sánchez-Pintos, P., Couce, M. L., Gaisford, S. & Basit, A. W. (2019c) Automated therapy preparation of isoleucine formulations using 3D printing for the treatment of MSUD: First single-centre, prospective, crossover study in patients. *Int J Pharm*, 567, 118497.
Gültekin, H. E., Tort, S. & Acartürk, F. (2019) An Effective Technology for the Development of Immediate Release Solid Dosage Forms Containing Low-Dose Drug: Fused Deposition Modeling 3D Printing. *Pharm Res*, 36(9), 128.
Hammond, M., Nunn, H., Rogers, G., Lee, H., Marghitoiu, A. L., Perez, L., Nashed-Samuel, Y., Anderson, C., Vandiver, M. & Kline, S. (2013) Identification of a leachable compound detrimental to cell growth in single-use bioprocess containers. *PDA J Pharm Sci Technol*, 67(2), 123-34.
Harinarayana, V. & Shin, Y. C. (2021) Two-photon lithography for three-dimensional fabrication in micro/nanoscale regime: A comprehensive review. *Optics & Laser Technology*, 142, 107180.
Hermabessiere, L., Receveur, J., Himber, C., Mazurais, D., Huvet, A., Lagarde, F., Lambert, C., Paul-Pont, I., Dehaut, A., Jezequel, R., Soudant, P. & Duflos, G. (2020) An Irgafos® 168 story: When the ubiquity of an additive prevents studying its leaching from plastics. *Science of The Total Environment*, 749, 141651.
Herrada-Manchón, H., Rodríguez-González, D., Alejandro Fernández, M., Suñé-Pou, M., Pérez-Lozano, P., García-Montoya, E. & Aguilar, E. (2020) 3D printed gummies: Personalized drug dosage in a safe and appealing way. *Int J Pharm*, 587, 119687.
Ibrahim, D., Broilo, T. L., Heitz, C., de Oliveira, M. G., de Oliveira, H. W., Nobre, S. M., Dos Santos Filho, J. H. & Silva, D. N. (2009) Dimensional error of selective laser sintering, three-dimensional printing and PolyJet models in the reproduction of mandibular anatomy. *J Craniomaxillofac Surg*, 37(3), 167-73.
Ibrahim, M., Barnes, M., McMillin, R., Cook, D. W., Smith, S., Halquist, M., Wijesinghe, D. & Roper, T. D. (2019) 3D Printing of Metformin HCl PVA Tablets by Fused Deposition Modeling: Drug Loading, Tablet Design, and Dissolution Studies. *AAPS PharmSciTech*, 20(5), 195.
Ilyés, K., Kovács, N. K., Balogh, A., Borbás, E., Farkas, B., Casian, T., Marosi, G., Tomuță, I. & Nagy, Z. K. (2019) The applicability of pharmaceutical polymeric blends for the fused deposition modelling (FDM) 3D

technique: Material considerations-printability-process modulation, with consecutive effects on in vitro release, stability and degradation. *Eur J Pharm Sci*, 129, 110-123.
Infanger, S., Haemmerli, A., Iliev, S., Baier, A., Stoyanov, E. & Quodbach, J. (2019) Powder bed 3D-printing of highly loaded drug delivery devices with hydroxypropyl cellulose as solid binder. *International Journal of Pharmaceutics*, 555, 198-206.
Jem, K. J. & Tan, B. (2020) The development and challenges of poly (lactic acid) and poly (glycolic acid). *Advanced Industrial and Engineering Polymer Research*, 3(2), 60-70.
Karavasili, C., Gkaragkounis, A., Moschakis, T., Ritzoulis, C. & Fatouros, D. G. (2020) Pediatric-friendly chocolate-based dosage forms for the oral administration of both hydrophilic and lipophilic drugs fabricated with extrusion-based 3D printing. *European Journal of Pharmaceutical Sciences*, 147, 105291.
Katstra, W. E., Palazzolo, R. D., Rowe, C. W., Giritlioglu, B., Teung, P. & Cima, M. J. (2000) Oral dosage forms fabricated by Three Dimensional Printing™. *Journal of Controlled Release*, 66(1), 1-9.
Kempin, W., Domsta, V., Grathoff, G., Brecht, I., Semmling, B., Tillmann, S., Weitschies, W. & Seidlitz, A. (2018) Immediate Release 3D-Printed Tablets Produced Via Fused Deposition Modeling of a Thermo-Sensitive Drug. *Pharm Res*, 35(6), 124.
Kempin, W., Franz, C., Koster, L.-C., Schneider, F., Bogdahn, M., Weitschies, W. & Seidlitz, A. (2017) Assessment of different polymers and drug loads for fused deposition modeling of drug loaded implants. *European Journal of Pharmaceutics and Biopharmaceutics*, 115, 84-93.
Kim, S. J., Lee, J. C., Ko, J. Y., Lee, S. H., Kim, N. A. & Jeong, S. H. (2021) 3D-printed tablets using a single-step hot-melt pneumatic process for poorly soluble drugs. *International Journal of Pharmaceutics*, 595, 120257.
Kjar, A. & Huang, Y. (2019) Application of Micro-Scale 3D Printing in Pharmaceutics. *Pharmaceutics*, 11(8).
Lee, A., Sudau, K., Ahn, K. H., Lee, S. J. & Willenbacher, N. (2012) Optimization of Experimental Parameters to Suppress Nozzle Clogging in Inkjet Printing. *Industrial & Engineering Chemistry Research*, 51(40), 13195-13204.
Leong, K. F., Wiria, F. E., Chua, C. K. & Li, S. H. (2007) Characterization of a poly-epsilon-caprolactone polymeric drug delivery device built by selective laser sintering. *Biomed Mater Eng*, 17(3), 147-57.
Li, K., Rogers, G., Nashed-Samuel, Y., Lee, H., Mire-Sluis, A., Cherney, B., Forster, R., Yeh, P. & Markovic, I. (2015) Creating a Holistic Extractables and Leachables (E&L) Program for Biotechnology Products. *PDA J Pharm Sci Technol*, 69(5), 590-619.
Limongi, T., Susa, F., Allione, M. & di Fabrizio, E. (2020) Drug Delivery Applications of Three-Dimensional Printed (3DP) Mesoporous Scaffolds. *Pharmaceutics*, 12(9), 851.
Liu, S., Zhao, P., Wu, S., Zhang, C., Fu, J. & Chen, Z. (2019) A Pellet 3D Printer: Device Design and Process Parameters Optimization. *Advances in Polymer Technology*, 2019, 5075327.
Maibohm, C., Silvestre, O. F., Borme, J., Sinou, M., Heggarty, K. & Nieder, J. B. (2020) Multi-beam two-photon polymerization for fast large area 3D periodic structure fabrication for bioapplications. *Scientific Reports*, 10(1), 8740.
Martin, V., Ribeiro, I. A., Alves, M. M., Gonçalves, L., Claudio, R. A., Grenho, L., Fernandes, M. H., Gomes, P., Santos, C. F. & Bettencourt, A. F. (2019) Engineering a multifunctional 3D-printed PLA-collagen-minocycline-nanoHydroxyapatite scaffold with combined antimicrobial and osteogenic effects for bone regeneration. *Materials Science and Engineering: C*, 101, 15-26.
Martinez, P. R., Goyanes, A., Basit, A. W. & Gaisford, S. (2017) Fabrication of drug-loaded hydrogels with stereolithographic 3D printing. *Int J Pharm*, 532(1), 313-317.
Melocchi, A., Uboldi, M., Cerea, M., Foppoli, A., Maroni, A., Moutaharrik, S., Palugan, L., Zema, L. & Gazzaniga, A. (2020) A Graphical Review on the Escalation of Fused Deposition Modeling (FDM) 3D Printing in the Pharmaceutical Field. *Journal of Pharmaceutical Sciences*, 109(10), 2943-2957.
Musazzi, U. M., Selmin, F., Ortenzi, M. A., Mohammed, G. K., Franzé, S., Minghetti, P. & Cilurzo, F. (2018) Personalized orodispersible films by hot melt ram extrusion 3D printing. *International Journal of Pharmaceutics*, 551(1), 52-59.
Muzaffar, A., Ahamed, M. B., Deshmukh, K., Kovářík, T., Křenek, T. & Pasha, S. K. K. (2020) Chapter 4 - 3D and 4D printing of pH-responsive and functional polymers and their composites, in Sadasivuni, K. K., Deshmukh, K. & Almaadeed, M. A. (eds), *3D and 4D Printing of Polymer Nanocomposite Materials*Elsevier, 85-117.
Norwood, D. L., Paskiet, D., Ruberto, M., Feinberg, T., Schroeder, A., Poochikian, G., Wang, Q., Deng, T. J., DeGrazio, F., Munos, M. K. & Nagao, L. M. (2008) Best practices for extractables and leachables in

orally inhaled and nasal drug products: an overview of the PQRI recommendations. *Pharm Res*, 25(4), 727-39.
Oh, B.-C., Jin, G., Park, C., Park, J.-B. & Lee, B.-J. (2020) Preparation and evaluation of identifiable quick response (QR)-coded orodispersible films using 3D printer with directly feeding nozzle. *International Journal of Pharmaceutics*, 584, 119405.
Okwuosa, T. C., Soares, C., Gollwitzer, V., Habashy, R., Timmins, P. & Alhnan, M. A. (2018) On demand manufacturing of patient-specific liquid capsules via co-ordinated 3D printing and liquid dispensing. *European Journal of Pharmaceutical Sciences*, 118, 134-143.
Oliveira, M., Santos, E., Araújo, A., Fechine, G. J. M., Machado, A. V. & Botelho, G. (2016) The role of shear and stabilizer on PLA degradation. *Polymer Testing*, 51, 109-116.
Ong, J. J., Awad, A., Martorana, A., Gaisford, S., Stoyanov, E., Basit, A. W. & Goyanes, A. (2020) 3D printed opioid medicines with alcohol-resistant and abuse-deterrent properties. *International Journal of Pharmaceutics*, 579, 119169.
Prasad, L. K. & Smyth, H. (2016) 3D Printing technologies for drug delivery: a review. *Drug Development and Industrial Pharmacy*, 42(7), 1019-1031.
Reddy Dumpa, N., Bandari, S. & M, A. R. (2020) Novel Gastroretentive Floating Pulsatile Drug Delivery System Produced via Hot-Melt Extrusion and Fused Deposition Modeling 3D Printing. *Pharmaceutics*, 12(1).
Rindelaub, J. D., Baird, Z., Lindner, B. A. & Strantz, A. A. (2019) Identifying extractable profiles from 3D printed medical devices. *PLOS ONE*, 14(5), e0217137.
Rycerz, K., Stepien, K. A., Czapiewska, M., Arafat, B. T., Habashy, R., Isreb, A., Peak, M. & Alhnan, M. A. (2019) Embedded 3D Printing of Novel Bespoke Soft Dosage Form Concept for Pediatrics. *Pharmaceutics*, 11(12).
Saleh, E., Zhang, F., He, Y., Vaithilingam, J., Fernandez, J. L., Wildman, R., Ashcroft, I., Hague, R., Dickens, P. & Tuck, C. (2017) 3D Inkjet Printing of Electronics Using UV Conversion. *Advanced Materials Technologies*, 2(10), 1700134.
Salmoria, G. V., Vieira, F. E., Muenz, E. A., Gindri, I. M., Marques, M. S. & Kanis, L. A. (2018) Additive Manufacturing of PE/fluorouracil/progesterone intrauterine device for endometrial and ovarian cancer treatments. *Polymer Testing*, 71, 312-317.
Sánchez-Guirales, S. A., Jurado, N., Kara, A., Lalatsa, A. & Serrano, D. R. (2021) Understanding Direct Powder Extrusion for Fabrication of 3D Printed Personalised Medicines: A Case Study for Nifedipine Minitablets. *Pharmaceutics*, 13(10).
Selimis, A., Mironov, V. & Farsari, M. (2015) Direct laser writing: Principles and materials for scaffold 3D printing. *Microelectronic Engineering*, 132, 83-89.
Sjöholm, E. & Sandler, N. (2019) Additive manufacturing of personalized orodispersible warfarin films. *International Journal of Pharmaceutics*, 564, 117-123.
Solanki, N. G., Tahsin, M., Shah, A. V. & Serajuddin, A. T. M. (2018) Formulation of 3D Printed Tablet for Rapid Drug Release by Fused Deposition Modeling: Screening Polymers for Drug Release, Drug-Polymer Miscibility and Printability. *Journal of Pharmaceutical Sciences*, 107(1), 390-401.
Stults, C. L., Ansell, J. M., Shaw, A. J. & Nagao, L. M. (2015) Evaluation of extractables in processed and unprocessed polymer materials used for pharmaceutical applications. *AAPS PharmSciTech*, 16(1), 150-64.
Tagami, T., Ito, E., Kida, R., Hirose, K., Noda, T. & Ozeki, T. (2021) 3D printing of gummy drug formulations composed of gelatin and an HPMC-based hydrogel for pediatric use. *International Journal of Pharmaceutics*, 594, 120118.
Tagami, T., Kuwata, E., Sakai, N. & Ozeki, T. (2019) Drug Incorporation into Polymer Filament Using Simple Soaking Method for Tablet Preparation Using Fused Deposition Modeling. *Biol Pharm Bull*, 42(10), 1753-1760.
Tamay, D. G., Dursun Usal, T., Alagoz, A. S., Yucel, D., Hasirci, N. & Hasirci, V. (2019) 3D and 4D Printing of Polymers for Tissue Engineering Applications. *Frontiers in Bioengineering and Biotechnology*, 7.
Tan, K. H., Chua, C. K., Leong, K. F., Cheah, C. M., Cheang, P., Abu Bakar, M. S. & Cha, S. W. (2003) Scaffold development using selective laser sintering of polyetheretherketone–hydroxyapatite biocomposite blends. *Biomaterials*, 24(18), 3115-3123.
Trenfield, S. J., Goyanes, A., Telford, R., Wilsdon, D., Rowland, M., Gaisford, S. & Basit, A. W. (2018) 3D printed drug products: Non-destructive dose verification using a rapid point-and-shoot approach. *International Journal of Pharmaceutics*, 549(1), 283-292.

Uddin, M. J., Scoutaris, N., Klepetsanis, P., Chowdhry, B., Prausnitz, M. R. & Douroumis, D. (2015) Inkjet printing of transdermal microneedles for the delivery of anticancer agents. *International Journal of Pharmaceutics*, 494(2), 593-602.
Wang, J., Goyanes, A., Gaisford, S. & Basit, A. W. (2016) Stereolithographic (SLA) 3D printing of oral modified-release dosage forms. *International Journal of Pharmaceutics*, 503(1), 207-212.
Wang, J., Liu, Y., Zhang, X., Rahman, S. E., Su, S., Wei, J., Ning, F., Hu, Z., Martínez-Zaguilán, R., Sennoune, S. R., Cong, W., Christopher, G., Zhang, K. & Qiu, J. (2021) 3D printed agar/ calcium alginate hydrogels with high shape fidelity and tailorable mechanical properties. *Polymer*, 214, 123238.
Wei, C., Solanki, N. G., Vasoya, J. M., Shah, A. V. & Serajuddin, A. T. M. (2020) Development of 3D Printed Tablets by Fused Deposition Modeling Using Polyvinyl Alcohol as Polymeric Matrix for Rapid Drug Release. *Journal of Pharmaceutical Sciences*, 109(4), 1558-1572.
Wu, B. M., Borland, S. W., Giordano, R. A., Cima, L. G., Sachs, E. M. & Cima, M. J. (1996) Solid free-form fabrication of drug delivery devices. *Journal of Controlled Release*, 40(1), 77-87.
Xu, X., Robles-Martinez, P., Madla, C. M., Joubert, F., Goyanes, A., Basit, A. W. & Gaisford, S. (2020) Stereolithography (SLA) 3D printing of an antihypertensive polyprintlet: Case study of an unexpected photopolymer-drug reaction. *Additive Manufacturing*, 33, 101071.
Yan, J., Wang, Y., Zhang, X., Zhao, X., Ma, J., Pu, X., Wang, Y., Ran, F., Wang, Y., Leng, F. & Zhang, W. (2019) Snakegourd root/Astragalus polysaccharide hydrogel preparation and application in 3D printing. *International Journal of Biological Macromolecules*, 121, 309-316.
Yu, D.-G., Branford-White, C., Ma, Z.-H., Zhu, L.-M., Li, X.-Y. & Yang, X.-L. (2009) Novel drug delivery devices for providing linear release profiles fabricated by 3DP. *International Journal of Pharmaceutics*, 370(1), 160-166.
Zhou, L., Ramezani, H., Sun, M., Xie, M., Nie, J., Lv, S., Cai, J., Fu, J. & He, Y. (2020) 3D printing of high-strength chitosan hydrogel scaffolds without any organic solvents. *Biomaterials Science*, 8(18), 5020-5028.

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