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Review Article

Toward a Novel Pharmacology and Therapeutic Understanding of Brazilian Propolis: A Meta-Analytical Approach

Arlik Rafael Santiago de Sousa[1]*, Sabrina Zelice Cruz de Moraes[2], Arleu Barbosa Viana-Júnior[3], Edílson Divino de Araujo[1]

ABSTRACT

This review article aims to use a meta-analytical approach to systematize and compare the main effects of green and red propolis, as determined through biological assays, in order to inform future preclinical tests and drug development for this natural product. A search for the full spectrum of their biological properties and possible pharmacological and medical applications found that propolis has anti-inflammatory, antioxidant, antiviral, antibacterial, antifungal, antiparasitic, anti-proliferative, anticarcinogenic, antinociceptive and neuroprotective effects, arising mainly from polyphenolic compounds. In order to identify and retrieve the most important published literature in this field in the last ten years a database search (LILACS, PubMed, Scopus and Web of Science) was performed. The MeSH terms and free text words used were: green propolis, red propolis, anti-inflammatory, antioxidant, antimicrobial, antiviral, antibacterial, anti-fungal, anti-parasitic, anti-carcinogenic, anti-proliferative, cytotoxicity, analgesic, anti-nociceptive, neuroprotective and neuroregenerative as well as their respective combinations. The meta-analysis outcomes showed that red propolis is superior to green propolis for healing, cytotoxic, antiparasitic and antibacterial applications; however, there are no significant differences between them as regards antifungal, antioxidant and anti-inflammatory activities. Furthermore, it was found that red propolis contains greater amounts of flavonoids than green propolis, and that the supercritical extraction method was better in relation to phenolic acid yields, whereas the ethanolic method was better in respect of flavonoids. The key findings of this study can help to direct future work on this natural product in the pharmacological and medical fields.

Key words: Meta-analysis, Medical applications, Natural product, Polyphenolic compounds, Propolis.

Arlik Rafael Santiago de Sousa[1]*, Sabrina Zelice Cruz de Moraes[2], Arleu Barbosa Viana-Júnior[3], Edílson Divino de Araujo[1]

[1]Department of Biology, Federal University of Sergipe, Marechal Rondon Avenue, S/N, Rosa Elze, São Cristóvão-SE CEP, BRAZIL.
[2]Department of Physiology, Federal University of Sergipe, Marechal Rondon Avenue, S/N, Rosa Elze, São Cristóvão-SE CEP, BRAZIL.
[3]Graduate Program in Biodiversity and Evolution, Coordination of Zoology, Paraense Emílio Goeldi Museum (MPEG), Belém, Pará, BRAZIL.

Correspondence

Dr. Arlik Rafael Santiago de Sousa,

Department of Biology, Federal University of Sergipe, Marechal Rondon Avenue, S/N, Rosa Elze, São Cristóvão-SE CEP: 49.000-100, BRAZIL.

Phone no : +55 (79) 99828-2105

E-mail: arlikrafael@hotmail.com

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INTRODUCTION

Propolis is a natural product derived from bees, with a resinous consistency, whose quality and quantity aspects depends largely on the bee flora, region and period in which it was collected.[1,2] It has been used by diverse civilizations since ancient times because of its therapeutic and medicinal properties. It is a complex compound composed of tree resins, beeswax, essential oils and pollen modified by the action of enzymes present in bee saliva.[3] Among the more than 300 metabolites that have been found in propolis, flavonoids, phenolic acids and terpenes stand out.[4]

Propolis, like other natural products, has faced issues in relation to its collection, extraction, concentration and standardization to ensure its most effective use in medical applications.[5] The aforementioned effect of seasonal variation on its chemical composition is a serious obstacle for drug development. Nevertheless, it is believed that phenolic acids and flavonoids derived from natural products such as propolis can play a major role in modulating human physiology.[6,7]

These bioactive compounds extracted from Brazilian propolis have shown potential as neuroregenerative, immunomodulatory and anti-inflammatory agents in animal models and have emerged as a promising source of therapeutic innovation.[8-11]

Furthermore, as an attempt of avoiding or minimizing commonly used drugs side effects, Brazilian propolis can be a promising target that has not yet been fully explored and understood.[12,13] Several polyphenolic compounds within red and green varieties have already begun to have their pathways traced through living systems,[14-16] but any differences between the green and red varieties, the best method of extraction for each one as well as the best variety to use in each clinical case is still to be fully explored.

To answer these questions and help maximize the benefits of their use in further preclinical tests and drug development studies a meta-analytical approach has been used to compare the properties of the red and green varieties to better understand their: (1) medical applications, (2) extraction methods, (3) chemical profiles and (4) study models used.

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MATERIALS AND METHODS

Literature search and selection criteria

In order to identify and retrieve the most important published literature in this field in the last decade a database search (LILACS, PubMed, Scopus and Web of Science) was performed. The MeSH terms and free text words used were: green propolis, red propolis, anti-inflammatory, antioxidant, antimicrobial, antibacterial; antifungal, antiviral, antiparasitic, anticarcinogenic, antiproliferative, cytotoxicity, analgesic, antinociceptive, neuroprotective and neuroregenerative as well as their respective combinations. The search terms used as wild cards to achieve a meta-analytical approach was propolis compar* (red AND green); propolis* (green AND red extract). Studies that did not present data about collection (botanical origin, locality or season), extraction (methanolic, hydroalcoholic, subfractions, aqueous or supercritical), or did not describe biological tests were excluded from the analysis. The main inclusion criterion was medical applications using the red and green brazilian propolis varieties. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) were used as reviewing guidelines and based on it an illustration was made to report our results (Figure 1).

Statistical data

We used the natural log of the response ratio (LnRR) to estimate the general effects of interest for our meta-analysis. Thus, the natural log of the response ratio is LnRR = ln ($\bar{Y}1$ / $\bar{Y}2$), where $\bar{Y}1$ is the mean of the parameter associated with red propolis and $\bar{Y}2$ is the mean of the parameter associated with green propolis. The use of LnRR is ideal when we want to compare the magnitudes of two means with the same signal and when we do not have the data of variance and the sample size of all the individual comparisons;[49] however, for those data where we can extract information about variance and sample size, the variance of the individual effect size is $\bar{v}$LnRR = $\sigma^2 1 / n1\bar{Y}^2 1 + \sigma^2 2 / n2\bar{Y}^2 2$, where $\sigma^2 1$ and $\sigma^2 2$ is the standard deviation of the mean associated with red and green propolis, respectively, and n1 and n2 are the sample sizes associated with red and green propolis, respectively. Positive effect values, when LnRR> 0, indicate that the red propolis has superior activity compared to the green propolis and negative effect values, when LnRR <0, indicate that the green propolis has superior activity compared to the red propolis; however, some responses found in the individual studies contained negative effect sizes (e.g., cell death) that were associated with a positive effect of red propolis and vice versa. Thus, these individual effect sizes were converted to positive or negative values so that there were no misinterpretations in the general effect of the meta-analysis. After calculating all the individual effects, we used linear random effects models to calculate the cumulative effects of each medical application used in the study (antifungal, anti-inflammatory, antibacterial, antioxidant, antiparasitic, cytotoxic and healing). These models assume that true effect sizes may vary across studies, given the variety of methods used within the studies.[17,18] We also used mixed-effect models for analysis of the moderator variables (extract type and model study),[18] because this model assumes that the differences between studies within a class are determined by sampling errors and random variation. For studies that involve the synthesis of data, the principles of mixed-effect models are usually fulfilled, and therefore, these are preferably used.[18,19] As we do not have all the data of mean variance information and sample number, we used unweighted meta-analysis models, since these models become equal to a linear model,[20] using the lmer function of the package lme4 for software R.[21,22] We calculated the 95% confidence intervals based on estimated effect sizes from a bias-corrected bootstrap approach using resampling tests generated from 10,000 simulations.[23] We assume that the cumulative effect size was considered significant if the bootstrap confidence intervals did not overlap zero.[24-26]

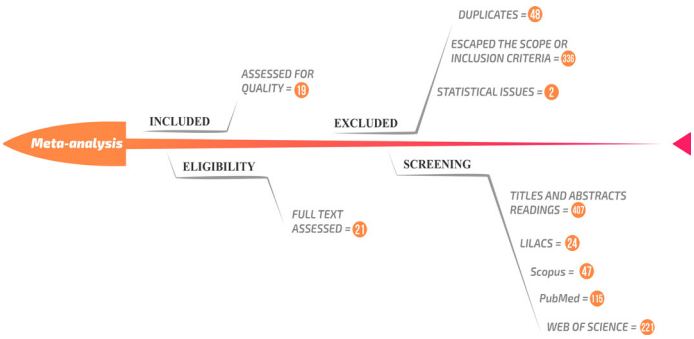

Figure 1: Search and selection screening diagram.

Publication bias

As suggested by Nagawama *et al.* 2017 we ran models both with and without data with sampling variance information since more than 50% of our data does not have variance information included. Models with different weights were then performed and the results of the comparison of all the models were included in the supplementary material to help us in further discussion in the present study; however, we only present the data based on the unweighted meta-analysis models. For each effect tested, we also calculated fail-safe numbers, which indicate how many non-significant, unpublished, or missing studies would need to be added to the sample to change its results from significant to nonsignificant. As a rule, results are considered robust if the fail-safe number exceeds 5*n**10, where *n* is the number of comparisons.[27] In order to assess publication bias, we used graphical assessment tools such as funnel plots and scatter plots. The funnel plot helped to detect funnel asymmetry, which can be caused by publication bias, and the scatter plots to show temporal trends in effect size or relationship between effect size and impact factors of journals.

RESULTS

Qualitative results

A total of 19 articles were found that met the inclusion criteria used in our meta-analysis (Figure 1). The temporal amplitude publications varied between the years of 2007 and 2018 and the range of the impact factors of the journals that published the articles was between 0.27 and 4.41, with an average of 2.84. From the 19 articles obtained, a total of 361 comparisons were extracted. Of these comparisons, more than 50% were associated with the antibacterial and cytotoxic activity of propolis: 26.6% (96 comparisons, in six trials) and 24% (87 comparisons in six trials), respectively. In respect of the other comparisons, 16% were related to the antioxidant effect (58 comparisons in five trials), 14.4% the anti-inflammatory effect (52 comparisons in three trials) and 8.3% on the antifungal effect (30 comparisons in two trials). Antiparasitic and healing applications accounted for approximately 10% of total comparisons, 25 comparisons in two trials and 13 comparisons in one trial, respectively. When we evaluated the comparisons according to the study models used to test the effects of propolis, we found that 59.8% used culture media (216 comparisons), while 26% performed chemical assays (94 comparisons) accounting for a total of 85% of the *in vitro* assays against only 14% *in vivo* models (51 comparisons). As for the types of extracts used to test the effects of propolis, 89% preferred the use of ethanolic extraction when compared to supercritical extraction.

Quantitative results

We observed a significant greater overall effect for red propolis compared to green propolis (LnRR = 0.37, bootstrap CI = 0.04 to 0.70), which shows that red propolis is 37% higher in its attributes than green propolis (Figure 2).

The evaluation of the effects of propolis in medical applications showed that red propolis is superior to green propolis in its antibacterial (LnRR = 0.83, bootstrap CI = 0.30 to 1.35), antiparasitic (LnRR = 0.94, CI bootstrap = 0.62 to 1.25), cytotoxic (LnRR = 0.68, CI bootstrap = 0.08 to 1.29) and healing (LnRR = 0.15, CI bootstrap = 0.03 to 0.26) activities. However, red and green propolis had no significant differences in antifungal (LnRR = -0.25, bootstrap CI = -2.31 to 1.78), anti-inflammatory (LnRR = 0.01, bootstrap CI = -2.31 to 1.78) and antioxidant (LnRR = 0.33, bootstrap CI = -0.16 to 0.82) activities (Figure 3).

When we evaluated the moderators (Table 1), we could only infer responses for the anti-inflammatory and antibacterial activities in the study model used. Thus, for the anti-inflammatory effect, the tested study model continues without significant effect for both types of propolis (Figure 4).

When we observed the type of extract used in the tests our inferences are for the antibacterial and antioxidant applications. For the antibacterial activity, only the ethanolic extract had a positive and significant effect (LnRR = 0.87, bootstrap CI = 0.23 to 1.50), while there were no differences in the supercritical extract between the two types of propolis (Figure 4). In respect of antioxidant activity, there was no difference in effect between the extracts.

Assessment of publication bias

Fail-safe numbers for overall (1,285,329 studies), antibacterial (707 studies), cytotoxic (32,817 studies), and healing (1273 studies) effects were large relative to the number of independent comparisons included in the meta-analysis (361 studies, 96 studies, 87 studies, and 13 studies, respectively), indicating the strength of our results; however, our result for antiparasitic activity is not robust enough to infer conclusions about

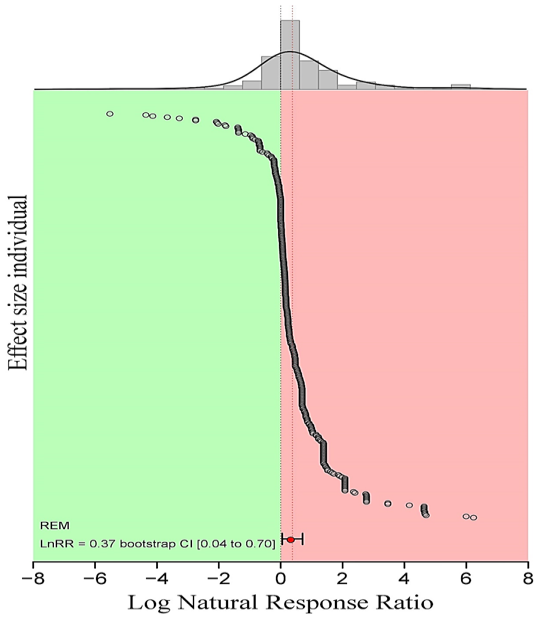

Figure 2: Random-effects model forest plot showing 361 effect size estimates based on logarithmic natural of response ratio for the 19 studies available. The closed red circle indicates the mean effect size with 95% confidence intervals based on bootstrap. The vertical black dotted line represents zero effect and the vertical red dotted line represents mean effect.

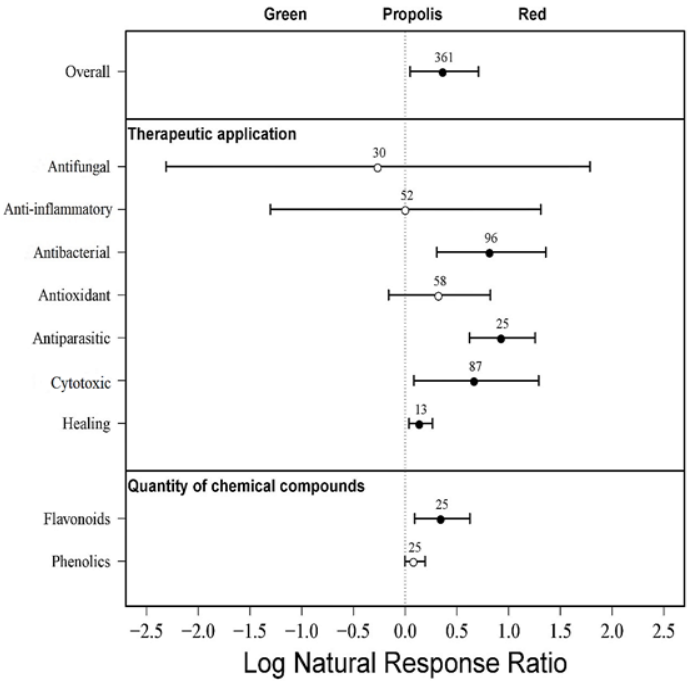

Figure 3: Effect-size plot from the linear random-effects model indicating the direction and magnitude of the effect of propolis for each therapeutic application and quantity of chemical compounds. The cumulative effect size is reported for each effect measured with its 95% confidence intervals, and effects are significant if confidence intervals do not overlap with zero. Superscript numbers indicate the number of independent comparisons for each effect. Positive effect sizes indicate that the red propolis has superior activity compared to the green propolis and negative effect sizes indicate that the green propolis has superior activity compared to the red propolis.

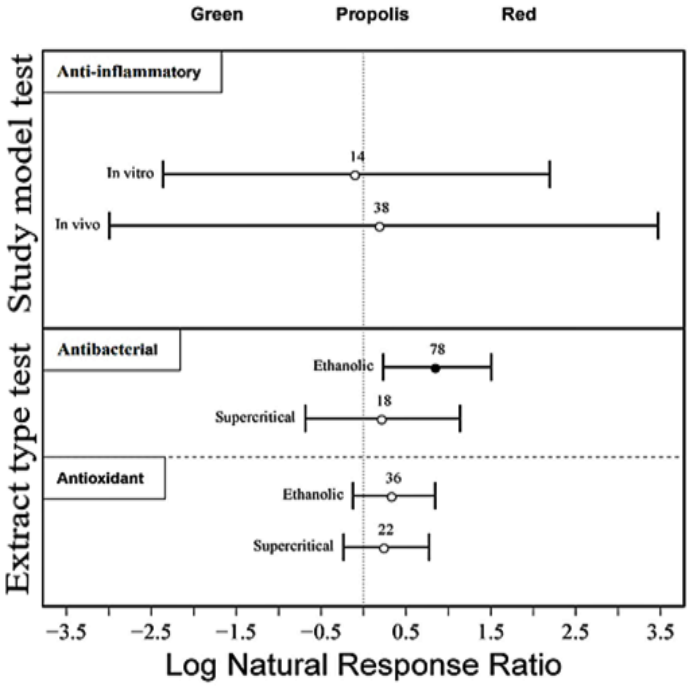

Figure 4: Effect-size plot from the linear mixed-effects model considering study model and extract type as fixed effects (moderator). The cumulative effect size is reported for each effect measured with its 95% confidence intervals, and effects are significant if confidence intervals do not overlap with zero. Superscript numbers indicate the number of independent comparisons for each effect.

Table 1: Main metadata moderators for the meta-analytical intervention.

Extract type	Study model	Medical applications	Action mechanisms	Ref
Ethanolic	*In vitro*	Antioxidant	*Through electron donation and stabilization of free radicals- REDOX balance	[29]
Ethanolic	*In vitro*	Antioxidant	*Through electron donation and stabilization of free radicals- REDOX balance	[30]
Ethanolic	*In vitro*	Cytotoxic	*Induced apoptosis via activation of TP53, CASP3, BAX, P21 signaling, and downregulating of BCL2 and BCL-XL	[26]
Ethanolic and Supercritical	*In vitro*	Antioxidant/ Antibacterial/ Antiparasitic	*By increasing the membrane permeability thus inhibiting bacterial and parasitic motility, provoking lysis and eventual cell death	[58]
Ethanolic	*In vitro*	Antibacterial	*Bacterial cell wall destruction by disorganizing the cytoplasm and causing cellular lysis	[59]
Ethanolic	*In vitro*	Antifungal	*Disruption of yeast cell wall affecting internal metabolism	[60]
Ethanolic	*In vitro*	Cytotoxic/ Antibacterial/ Antioxidant	*Bacterial cell wall destruction by disorganizing the cytoplasm and causing cellular lysis	[61]
Ethanolic and Supercritical	*In vitro*	Cytotoxic/ Antibacterial/ Antioxidant	*Inhibition of bacterial RNA polymerase which may also act on the local microbial membrane or cell wall, causing structural and functional damage	[54]
Ethanolic	*In vitro*	Cytotoxic/ Antibacterial	*Destabilization and permeabilization of the cytoplasmatic membrane, protein denaturation and inhibition of extracellular enzymes	[32]
Ethanolic	*In vitro*	Anti-inflammatory/ Cytotoxic	*Immunomodulation by stimulating or inhibiting the production of certain cytokines (TNF-α, IL-1β, IL-6)	[62]
Ethanolic	*In vitro*	Cytotoxic	*Inhibition of lipid peroxidation and angiogenesis	[37]
Ethanolic	*In vitro*	Antibacterial	*Inhibition of nucleic acid synthesis, affecting cytoplasmic membrane function, and energy metabolism	[63]
Ethanolic	*In vitro*	Cytotoxic	*Apoptosis by stimulating caspase expression	[64]
Ethanolic	*In vivo*	Healing	*Stimulates the production of keratinocytes and the production of FGF by macrophages	[65]
Ethanolic	*In vivo*	Anti-inflammatory	*Immunomodulating by downregulating pro-inflammatory cytokines, chemokines and angiogenic factors	[31]
Ethanolic	*In vitro*	Anti-inflammatory	*Inhibits the activity of NF-κB by inhibiting the degradation of IκB	[66]
Ethanolic	*In vitro*	Antioxidant	*Scavenging ROS and NOS species	[67]
Ethanolic	*In vitro*	Antifungal	*By altering the permeability of the membrane and, therefore, affecting metabolism leading to eventual cell death	[68]
Ethanolic	*In vitro*	Anti-parasitic	*Inducing macrophage activation, leading to production of cytokines and reactive nitrogen intermediates engaged in the killing of intracellular parasites	[69]

*The mechanisms of action were based on information given in the discussion section of the articles

the positive effect of red propolis. The fail-safe numbers estimated 37 comparisons to change our result from significant to non-significant, indicating a publication bias, a phenomenon known as the 'file-drawer problem'.[28] Our graphical analyses also show that our results show little bias and in general we have robust inference data. The funnel plot demonstrates a cloud of funnel shaped dots and as expected a larger variation of the effect sizes in the smaller sample sizes (Figure 5A). The scatter plots of the effect size ratio and year of publication (Figure 5B) and impact factor (Figure 5C) reinforce the low bias found in our analyses since the measurements of the individual effects for propolis are not influenced by the time of publication or even by the quality of the journal in which it was published (Figure 5).

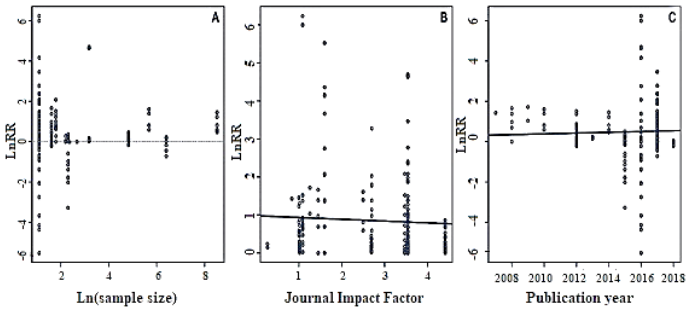

Figure 5: Multiple plots representing sensitivity analysis for testing for publication bias. A) Funnel plot showing the effect sizes over sample sizes where studies with low sample sizes are expected to show greater variability compared to studies with high sample sizes. B) Scatter plot showing the relationship between effect size and impact factors of journals, indicating that the magnitudes of effect sizes (the absolute values of LnRR) no tend to be published in higher impact journals. C) Scatter plot showing a temporal trend in effect size (LnRR) across years.

DISCUSSION

Several studies[29-33] have already reported that green and red propolis are more effective in terms of their bioactive properties than other propolis varieties; a fact, therefore, that should not be ignored in future medical exploitation and drug design for this natural product. Our study found that red propolis is superior to green propolis in respect of healing, antibacterial, antiparasitic and cytotoxic applications, matching studies[34-38] that also show red propolis to have better efficacy on healing, antiparasitic, antibacterial or cytotoxic properties when compared to another varieties, commonly used drugs and control groups; however, in terms of its antioxidant, anti-inflammatory or antifungal properties, there is no difference between them, with the green propolis being as good as the red propolis.

Our results also show that red propolis has a greater quantity of flavonoids than green propolis, but no significant differences were found between the two types in terms of phenolic acids. Thus, the superiority of red propolis in medical applications could be explained by the greater number of compounds, mainly flavonoids, and their synergistic effects with phenolic acids in modulating human physiology.[39] Since there are no significant differences between the phenolic acids present in both varieties, and given the fact they have shown the same potentials in relation to antioxidant activity, our data are supported by studies that show a correlation between phenolic acids and this activity.[40,41]

The absence of statistical differences and the similar potential of both the red and green varieties in these two applications (anti-inflammatory and antioxidant) corroborates the cause-and-effect relationship between free radicals and inflammatory process, in other words, both varieties when performing their antioxidant action are deeply engaged in scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS). Therefore, as demonstrated in other studies,[42,43] the bioactive compounds present in propolis act by inhibiting the cascade of events that leads to inflammation - mainly involved in immune cell recruiting- and oxidative damage - redox balance. On the other hand, in respect of cytotoxic action such molecules act to induce the formation of ROS/RNS and consequently cell death through apoptosis.[44]

These studies (Table1) have shown, therefore, that some of the phenolics and flavonoids present in propolis act specifically on mitochondrial metabolism, inhibiting or inducing ROS/RNS by modulating mitochondrial membrane potential maintenance, electron transport chains, ATP synthesis, triggering cell death and even epigenetic factors.[45,46] A number of studies[47-49] can allow us to infer that when antioxidant activity occurs in a synergistic way with either anti-inflammatory or cytotoxic activity, a third event may appear that could be analgesic, neuroprotective or antiproliferative.

It is important to note that this meta-analytical approach also reveals a lack of animal studies in the propolis field, despite the need for this type of testing given the increasing demand for novel drugs and therapeutic approaches as well as the complex interactions between human metabolites and the chemical entities present in natural products.[50] On the other hand, this study also reinforces the need to thoroughly investigate primary data since the biological pathways of some of the chemical entities have already been traced in mammal physiological systems and can be simulated *in silico,* as demonstrated in the study by Luechtefeld *et al*.[51] thereby preventing redundant studies that would unnecessarily use more *in vivo* models.

Some studies have pointed out that supercritical extraction is a more green method since it is less toxic than ethanolic or even methanolic methods;[52,53] however, these last two methods have been shown to be better to solubilize polyphenols compared to supercritical extraction.[54] Our study found that when supercritical extraction was compared with ethanolic extraction, it only demonstrated a superior effect in relation to antibacterial activity.[55] In respect of polyphenols, studies corroborate our findings that the supercritical extraction method was better for phenolic acids while the ethanolic extract showed better results with flavonoids.[56] The main advantages of supercritical extraction are its use of a non-toxic solvent (CO_2) and the low extraction temperature that preserves thermo-sensitive biomolecules such as phenolics acids and flavonoids.[57]

CONCLUSION

To the best of our knowledge, this study quantitatively confirms the effects of red and green propolis and determines some of their sources of variation for the first time. These results synthesize important knowledge about the potential therapeutic use of propolis and demonstrate which chemical and methodological factors determine the quality of the results in this research field. Several patterns emerge in this study and open research lines and possible applications that should be followed in respect of this natural product.

The meta-analysis reveals that red propolis is superior to green propolis in a number, but not in all, therapeutic applications. Our study suggests that in respect of healing, cytotoxic, antiparasitic and antibacterial applications red propolis is more effective than green propolis. On the other hand, for antifungal, antioxidant and anti-inflammatory applications there is no difference between them, indicating that one is as good as the other thus informing future studies on their use in pharmacology and experimental therapeutics.

Some of the most important findings of the study that can help to inform and direct future research are in relation to: (1) which variety should be used in each clinical case; (2) what are the best extraction methodologies for each purpose, whether in respect of flavonoids or phenolic acids; (3) the chemical differences between the varieties - suggesting that the class of flavonoids found in red propolis are responsible for its superiority; (4) the importance of new experimental designs in pre-clinical tests of this natural product; (5) the lack of animal studies in this area; (6) how costs can be reduced by avoiding redundant tests and focusing on the most productive areas for each variety.

We should emphasize that we need to better understand the different bioactive properties present in the two varieties. As well as the underlying synergistic effects on propolis mechanisms of action to achieve the full spectrum of their applications and experimental designs that maximizes the efficacy of their therapeutic aspects. Lastly, more *in vivo* studies are

needed to understand the intricate role played by polyphenols in human physiological systems.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ROS/RNS: Reactive oxygen species/ Reactive Nitrogen species; **LNRR**: Log natural response ratio; **CI:** Confidence Interval.

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