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EXPLORING THE THERAPEUTIC POTENTIAL OF PROPOLIS: INSIGHTS INTO ITS CHEMICAL PROFILE AND PHARMACOLOGICAL ACTIVITIES

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ABSTRACT

Propolis is a natural adhesive produced by honey bees. This study explored two extracts, methanolic and ethanolic, prepared through Soxhlet extraction and maceration, respectively. The antioxidative, antibacterial, and anticancer properties of these extracts were assessed. For quantification, flavonoid, phenolic, and tannin contents were determined in both extracts. The antioxidant properties of the extracts were evaluated using DPPH and ABTS radical scavenging assays. Antibacterial susceptibility test of the extracts was performed to evaluate their efficacy against gram-positive and gram-negative bacteria. Both extracts have also shown anti-cancer activity as well on leukemic cell line and hence can be taken into consideration for further pharmacological investigations along with their clinical applications.

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INTRODUCTION

Propolis, commonly referred to as bee glue, is a naturally occurring adhesive substance produced by honeybees (*Apis mellifera*). It is derived from a combination of mucilage, sap, and resin collected from different plant sources, including flower buds, tree barks, and leaves (Forma & Bryś, 2021; Iqbal *et al*., 2019; Zabaiou *et al*., 2019). The bees further enhance its composition by combining these resins with their salivary enzymes and beeswax (Przybyłek & Karpiński, 2019; Sforcin & Bankova, 2011; Silva *et al*., 2012). Propolis composition differs across continents, regions, and plant species, resulting in distinct differences. Propolis contains various chemical groups including phenolic esters, flavonoids, alkaloids, fatty acids, terpenes, aliphatic and aromatic acids, β-steroids, and alcohols. Notable alkaloids found in propolis are caffeic acid phenylethyl ester (CAPE), caffeic acid (CA), galangin, chrysin, p-coumaric acid, o-coumaric acid, pinocembrin, cinnamic acid, apigenin, kaempferol, and quercetin (Anjum *et al*., 2019; Noureddine *et al*., 2017; Stojanović *et al*., 2020; Zabaiou *et al*., 2017). Flavonoids are the main chemical components accountable for the medicinal properties of propolis, while terpenoids contribute to its aromatic characteristics (Zabaiou *et al*., 2017).

Propolis displays a wide range of biological traits, including antioxidant, antibacterial, antiviral, fungicidal,antiparasitic, immunomodulatory, antiproliferative, and anti-inflammatory properties, among numerous others (Alday *et al*., 2019; Catchpole *et al*., 2015; de Mendonça *et al*., 2015; Franchin *et al*., 2018, 2018; Freitas *et al*., n.d.; Martinello & Mutinelli, 2021; Przybyłek & Karpiński, 2019; Ramanauskienė & Inkėnienė, 2011; Ripari *et al*., 2021; Schnitzler *et al*., 2010). Additionally, Propolis and its constituents have anti-cancer properties, including antiproliferative and cytotoxic effects on cancer cells. They also have antiangiogenic effects and impact signalling pathways essential for cancer metastasis, demonstrating anti-metastatic qualities. Propolis can halt the cell cycle, trigger apoptosis, and decreases cancer cell viability, invasion, migration, and chemoresistance (Elumalai *et al*., 2022; Forma & Bryś, 2021; Iqbal *et al*., 2019; Santos *et al*., 2020; Schaller, 2010; Shahinozzaman *et al*., 2018; Tan & Hayati, 2017). The efficacy of chemotherapy using cytotoxic drugs can also be influenced by propolis (Sameni *et al*., 2021). Natural compounds, such as propolis, can also protect normal cells from the detrimentalimpacts of radiotherapy and chemotherapy. Furthermore, they can assist in reducing the severe repercussions of anticancer treatments (Motawi *et al*., 2016). Propolis in its raw form is unsuitable for direct analysis or treatment purposes. It is essential to extract the potent components by

dissolving them. Various solvents such as water, methanol, ethanol, hexane, acetone, chloroform, and dichloromethane have been utilized to extract these components (Przybyłek & Karpiński, 2019). In this study, dry powdered propolis was used to prepare two distinct types of extracts, namely ethanolic and methanolic extracts. These extracts were subjected to a series of assays to evaluate their antioxidative, antibacterial, and anticancer properties. The levels of particular biologically active compounds in the extracts were ascertained through quantitative assessment.

MATERIALS AND METHODS

Ethanolic Extraction: The ethanolic extraction process, based on protocol established by Maingi *et al*., involved dissolving 30g of propolis powder in 100ml of 70% ethanol. The solution was stirred every day and allowed to sit at room temperature for seven days before filtration and dried in an oven overnight. The final product was weighed, stored in a dark bottle in the refrigerator (Maingi *et al*. 2008).

Methanolic Extraction: Methanolic extraction was performed using a modified version of Paviani *et al*.'s method. 5 g of propolis was Soxhlet extracted for 6 hours at 60 °C with 200 mL of 70% methanol. The solution underwent filtration with Whatman No.1 paper, followed by drying, weighing, and subsequent storage in a dark bottle within a refrigerator (Paviani *et al*., 2013).

Determination of Total Phenolic Contents (TPC): The TPC was determined by using modified FC method by Ojha *et al*. The preparation of samples involved mixing stock extract solutions with FC reagent and incubating for 5 minutes. Following the addition of sodium carbonate, the solution was incubated in the absence of light for 45 minutes. The absorbance was then measured at 760 nm using a UV-Vis spectrophotometer. The phenolic content was determined using a calibration curve established using gallic acid standard, and the quantity of phenolic components in the extracts was determined as milligrams of GAE per gram of dry extract (Ojha *et al*., 2019).

Determination of Total Flavonoid Contents (TFC): The TFC was evaluated by the Aluminium chloride colorimetric method outlined by Price and Butler with minor adjustments. Distilled water, $NaNO_2$, $AlCl_3.6H_2O$, and NaOH were added to the extracts, then incubated and assessed at 510 nm. Flavonoid concentration was determined using a quercetin-based calibration curve and further expressed as milligrams of QE per gram of dry extract (Price & Butler, 1977).

Determination of Total Tannin Contents (TTC): The TTC was quantified using a modified version of Price and Butler's method. The extracts was mixed with water and 0.1M $FeCl_3$ and potassium ferricyanide, and subjected to 10 min at room temperature. Absorbance measurement was taken at 720 nm, and reagent blanks were prepared for each solvent. Tannin concentration was quantified using tannic acid calibration curve and expressed as TAE per gram of dry extract (Price & Butler, 1977).

DPPH radical scavenging activity assay: The antioxidant activity of PME and PEE extracts was evaluated by assessing their DPPH radical scavenging activity using a modified method from Rubab *et al*. 1ml of different concentrations (10-50 µg/ml) of the extracts were mixed with 1 ml DPPH solution and 200 µL Tris HCl, vortexed, kept in the darkness for 15 mins, and then absorbance at 517 nm was measured with the help of a spectrophotometer. Outcomes were expressed as DPPH radical scavenging activity percentage (Rubab *et al*., 2022).

ABTS radical scavenging assay: The extracts were evaluated for their ABTS radical scavenging potential using the assay outlined by Shalaby with minor modifications. Generation of ABTS radicals was done by mixing 7 mM ABTS with 2.45 mM $K_2S_2O_8$ and incubating in darkness for 14 h. Subsequent dilution of the solution was done with methanol until an absorbance of 0.700 ± 0.02 was obtained at 734 nm. 1 ml of samples at concentrations of 10 to 50 µg/ml were combined with the diluted ABTS solution and incubated for 7 min prior to measuring absorbance at 734 nm (Shalaby, 2013).

Antibacterial Susceptibility Testing (AST): Four bacterial cultures (Table 2), including both Gram-positive and Gram-negative bacteria (Table 2), were cultivated in a tryptic soy broth (TSB) medium (Nadeem *et al*., 2021). To assess the antibacterial susceptibility testing (AST) of both extracts, the Kirby-Bauer disk diffusion technique was used.

Table 1. Microbial makeup of bacterial elements used in antimicrobial activity assessment

Microorganism	Accession number	Strain
Streptococcus pyogens	ATCC 19615	Gram-positive
Methicillin-Resistant *Staphylococcus aureus* (MRSA)	ATCC 43300	Gram-positive
Escherichia coli	ATCC 25922	Gram-negative
Klebsiella pneumoniae	ATCC 13883	Gram-negative

Agar plates with 10 mm diameter paper disks soaked in 20 µL of extract were used for analysis. The disks were positioned onto culture plates and allowed to incubate for 24 h at temperatures between 25°C and 35°C. Distilled water-soaked disks were used as a negative control to prevent potential bacterial growth interference. Aseptic conditions were maintained in a laminar flow environment. The samples were analysed in triplicate, and outcomes are reported as average inhibition zone sizes in millimeters (Nadeem *et al*., 2022).

Anti-cancer activity: Anti-cancer activity was determined using the MTT cytotoxicity assay. On day 1, 10,000 cells per well were seeded in 96-well plate following which PME and PEE treatments were given on day 2. Along with PME and PEE treatments, standard drug treatments were also given. On the 3rd consecutive 10 µl MTT was added and kept for incubation for 2.5 hours and ultimately 100 µl DMSO was added in order to dissolve the formazan crystals. The absorbance was measured at 570 nm in Epoch plate reader (Desai *et al*., 2023; Joshi *et al*., 2024; Saad *et al*., 2011).

RESULTS AND DISCUSSION

Total Phenolic Content: Spectrophotometric analysis using the F-C method was utilized to quantify the total phenolic content (TPC) in the samples. Concentrations between 10 µg/L and 50 µg/L of Gallic acid (GA) were employed as the standard for developing the calibration curve. As illustrated in Figure 1, a gallic acid standard curve ($y = 0.0302x+0.2366$, $R^2 = 0.9725$) was obtained and the analysis outcomes, TPC of the extracts in milligrams of Gallic acid equivalent per gram of dry extract (mg GAE/g) are presented.

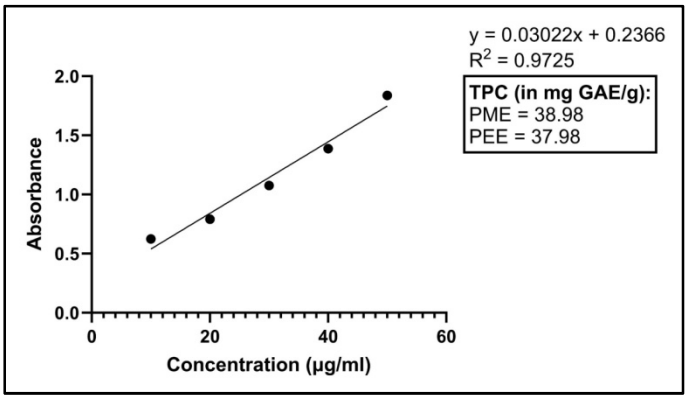

Figure 1. Concentration v/s absorbance standard curve & TPC of the extracts

The phenolic content in both samples was found to be comparable, with the methanolic extract (38.98 mg GAE/g) exhibiting a slightly higher level compared to the ethanolic extract (37.98 mg GAE/g). This indicated that the solvent used in the extraction process may also affect the phenolic yield. The broad range of biological activities exhibited by phenolic compounds, such as immunopotentiation, chemo preventive properties, and antitumor effects, is now widely recognized (Szliszka *et al*., 2011).

Total Flavonoid Content: The total flavonoid content (TFC) in the samples was quantified by applying the regression equation derived from the calibration curve. (y = 0.0003600x+0.5540, R^2= 0.9205), using quercetin as the standard.

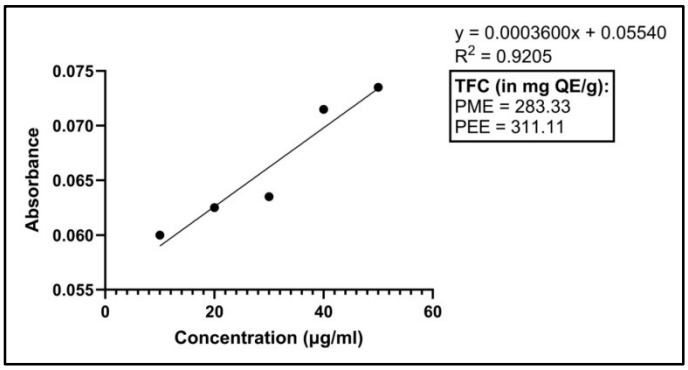

Figure 2. Concentration v/s absorbance standard curve & TFC of the extracts

The plotwas used to calculate the amount of flavonoids present in the samples, which were then calculated as milligrams of quercetin equivalents per gram of dry extract (mg QE/g) and are presented in Figure 2.The flavonoid content was found to be higher in the ethanolic extract in comparison to the methanolic extract, with a total flavonoid content (TFC) of 311.11 mg QE/g and 283.33 mg QE/g, respectively. Flavonoids exhibit various therapeutic advantages, including anticancer, antioxidant, antibacterial, antimalarial, antidiabetic, anti-inflammatory, and antiviral properties. Moreover, they demonstrate neuroprotective and cardioprotective effects (Ullah *et al*. 2020).

Total Tannin Content: The extracts' Total Tannin Content (TTC) was quantifiedusing the regression equation (y = 0.01606x+0.1779, R^2= 0.9298), with tannic acid as the standard.

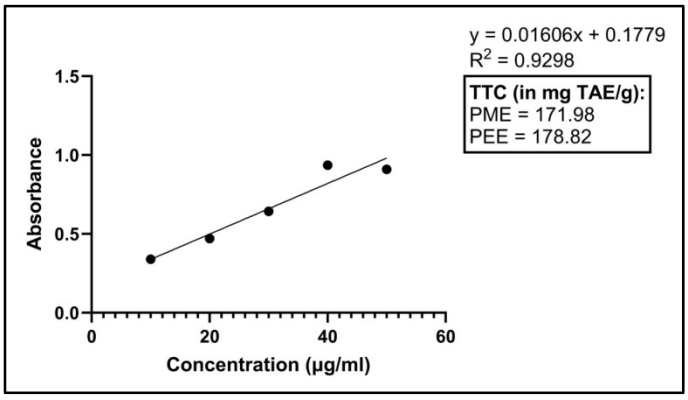

Figure 3. Concentration v/s absorbance standard curve & TTC of the extracts

The plot was used to calculate the amount of flavonoids in the extracts. This value was then converted to milligrams of tannic acid equivalents per gram of dry extract (mg TAE/g), as shown in Figure 3. Similar to flavonoids, tannin concentration was greater in the ethanolic extract. The tannin concentration in the methanolic extract was 171.98 mg TAE/g, while the ethanolic extract contained 178.82 mg TAE/g. These findings indicate that ethanol is a more effective solvent for the extraction of flavonoids and tannins. Research has indicated that tannins exhibit properties that can effectively combat both diarrhoea and microbial infections (Dall'Agnol *et al*., 2003; Mbagwu & Adeyemi, 2008; Siqueira *et al*., 2012; Tian *et al*., 2009). Tannins can also reduce the risk of cancer and neurodegenerative conditions such as cardiovascular diseases and Alzheimer's disease (Pizzi, 2021). Consequently, these natural compounds can be utilized as nutraceuticals to support healthy gut microbiota (Pizzi, 2021).

Antioxidant Capacity: Antioxidant activity was determined for both propolis extracts. The assessment of antioxidant property relied on the electron donation mechanism using DPPH assay and stabilization of ABTS following hydrogen donation (Kurek-Górecka *et al*., 2022). DPPH scavenging assay was performed using ascorbic acid as the reference standard (Figure 4). The percentage inhibition was determinedby employingequation 1:

$$\%\ inhibition = \frac{A_0 - A_1}{A_1} \times 100 \qquad \ldots\ldots. (1)$$

A_0 = Absorbance of control
A_1 = Absorbance of sample

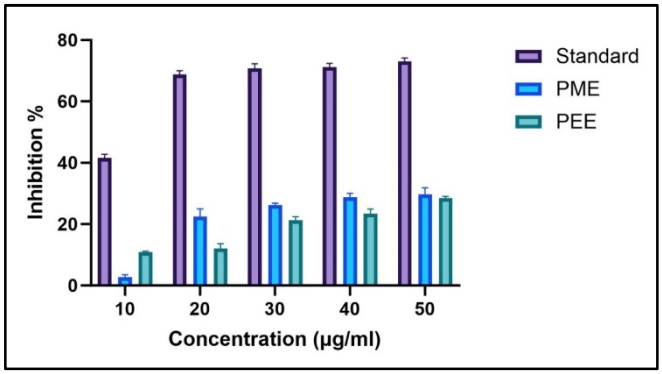

Figure 4. % Inhibition of DPPH radical (Here, standard = Ascorbic Acid)

Similarly, the ABTS assay was performed using ascorbic acid as the standard, and the percentage of inhibition was determined (Figure 5).

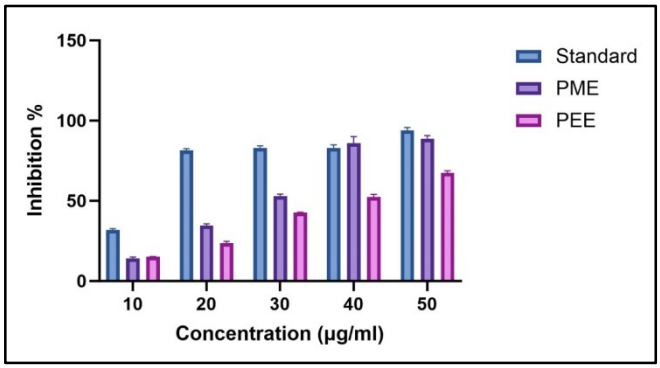

Figure 5. % Inhibition of ABTS radical (Here, standard = Ascorbic acid)

In both assays, PME prepared through Soxhlet extraction showed higher radical scavenging activity than PEE prepared through maceration. This indicates that the antioxidant potential of the extracts could be affected by the extraction method utilized, with Soxhlet extraction potentially facilitating more efficient extraction of antioxidant compounds. The antioxidant capacity of propolis plays a significant role in contributing to its biological advantages, such as chemoprevention. Propolis contains flavonoids, which possess strong antioxidant properties, enabling them to effectively neutralize free

radicals and safeguard the integrity of the cell membrane by preventing peroxidation of lipids(Daleprane & Abdalla, 2013; Kolankaya *et al*., 2002). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), along with various other factors, play a significant role in cellular aging and death in various health conditions, including arthritis, cardiovascular disease, Parkinson's disease, diabetes, cancer, and Alzheimer's disease(Butterfield & Dalle-Donne, 2012; Keeney *et al*., 2013; Kishida & Klann, 2007; Lipton *et al*., 2007; Matteo & Esposito, 2003). Propolis possesses the ability to diminish cellular concentrations of H_2O_2 and NO, potentially contributing to its anti-inflammatory properties(Tan-no *et al*., 2006).

Antibacterial Activity: The Kirby-Bauer disk diffusion technique was utilized to assess the antimicrobial effectiveness of the propolis extracts (methanolic and ethanolic) by measuring the zone of inhibition (mm) under *in vitro* conditions on solidified agar medium (Figure 6).

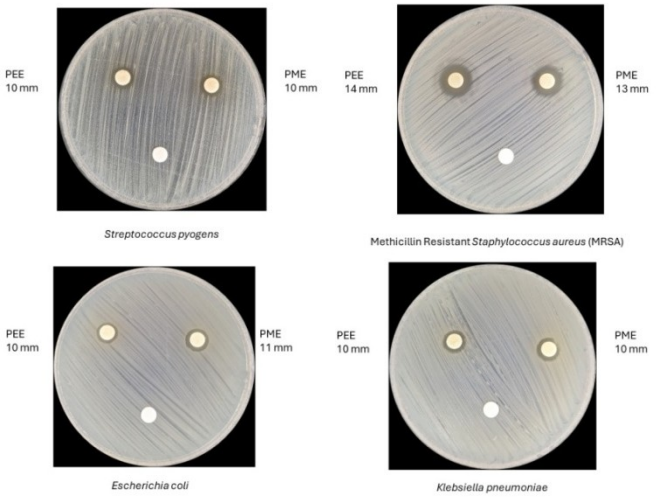

Figure 6. Zone of inhibition for PME and PEE for the respective bacterial strains

Table 2. The average zone of inhibition obtained for different bacterial strains

Bacteria	Zone of inhibition for PME (in mm)	Zone of inhibition for PEE (in mm)
Streptococcus pyogens	10	10
Methicillin Resistant Staphylococcus aureus (MRSA)	13	14
Escherichia coli	11	10
Klebsiella pneumoniae	10	10

The findings (Table 1) revealed that both PME and PEE exhibited antimicrobial activity against *Streptococcus pyogenes*, *methicillin-resistant Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Klebsiella pneumoniae*, with zones of inhibition indicating similar efficacies for both extracts against *S. pyogenes* and *K. pneumoniae* and a slight advantage of PEE over PME against MRSA. This highlights the potential of propolis extracts as effective antimicrobial agents against both Gram-positive and Gram-negative bacterial strains, even those that are resistant to antibiotics,like MRSA. By suggesting that the antimicrobial activity could be attributed to the intricate chemical makeup of propolis, including flavonoids and phenolic acids, the authors support the possible application of propolis as a substitute or complementary approach to traditional antimicrobials in managing bacterial infections, emphasizing the importance of further research to identify the active components and assess clinical efficacy.

Anti-cancer activity: The cytotoxic effects of propolis' methanolic and ethanolic extract along with two standard drug samples, Doxorubicin and Cytarabine was examined in THP-1 cell linethrough MTT assay and the half-maximal inhibitory concentration (IC_{50}) was obtained.

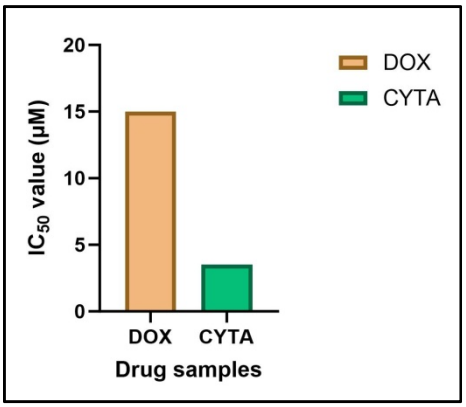

Figure 7. IC_{50} values (in µM) for standard drug samples used in THP1 cell line by MTT assay (Here, DOX = Doxorubicin and CYTA = Cytarabine)

Upon evaluation of the standard drugs used, a notable distinction in the IC_{50} values is observed, as Doxorubicin (14.99 µM) exhibits a greater IC_{50} value in contrast to Cytarabine (3.51 µM) (Figure 7).

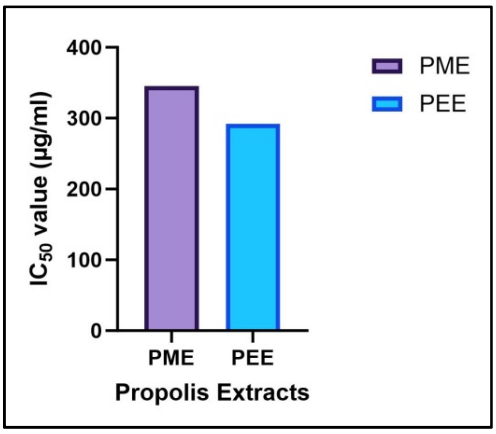

Figure 8. IC_{50} values (in µg/ml) for propolis extracts used in THP1 cell line by MTT assay

Thus, the study explores the anti-cancer/anti-leukemic activities of both propolis methanolic as well as ethanolic extracts on THP-1 cell line which is a monocytic acute myeloid leukemic cell line. Both PME and PEE extracts showed inhibitory concentrations of 345.27 µg/ml and 291.79 µg/ml (Figure 8) when compared to the standard drugs cytarabine and doxorubicin with concentrations of 3.51µM and 14.99 µM respectively (Franchi *et al*., 2012). However, the drugs cytarabine and doxorubicin are FDA approved drugs for administration in AML patients, hence were taken into consideration against PME and PEE. Nonetheless, it still becomes a necessity to check for further anti-cancer activities of these extracts using high-throughput techniques.

CONCLUSIONS

Propolis, a bee hive resinous product is an enriched source of phenols, flavonoids and tannins and also exhibits strong anti-oxidant potential along with anti-microbial consecutively anti-cancer activities. The present study can help as a stepping stone in the more distant research in the inquisition of propolis as a medicinal product. Nevertheless, a stronger and broader research in indeed needed for the use of propolis as a pharmacological product against diseases and disorders.

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Declaration of Interest statement: The authors state that there are no conflicts of interest to disclose.

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