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Original Article

Preformulation study and influence of DMSO and propylene glycol on the antioxidant action of isocoumarin paepalantine isolated from *Paepalanthus bromelioides*

João Paulo Loureiro Damasceno[a], Cristiane dos Santos Giuberti[a,b], Rita de Cássia Ribeiro Gonçalves[a,b], Rodrigo Rezende Kitagawa[a,b,*]

[a] *Programa de Pós-graduação em Ciências Farmaceuticas, Universidade Federal do Espírito Santo, Vitória, ES, Brazil*
[b] *Departamento de Ciências Farmaceuticas, Universidade Federal do Espírito Santo, Vitória, ES, Brazil*

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ABSTRACT

Coumarins are phenolic compounds and have various biological properties, including antioxidant activity. The isocoumarin paepalantine, isolated from of *Paepalanthus bromelioides* Silveira, Eriocaulaceae, exhibits a wide range of biological activities, including antimicrobial, anti-inflammatory, antioxidant and cytotoxic properties. Studies on paepalantine often use dimethylsulfoxide as a solvent. However the dimethylsulfoxide interferes with antimicrobial, cytotoxic and antioxidant assays. Thus, this study aims to evaluate alternative solvents for paepalantine and evaluate their potential to interfere with antioxidant assays ($ABTS^{\bullet+}$, $O_2^{\bullet-}$, HOCl). Of the selected solvents, propylene glycol had good solubility and remained stable throughout the study period. The results suggested that there is no interference from propylene glycol in antioxidant assays, while dimethylsulfoxide significantly interfered with the HOCl assay. The antioxidant assays showed that paepalantine demonstrated similar or even better antioxidant activity than Trolox. Thus, propylene glycol may be the solvent of choice for paepalantine, a compound that has significant biological potential.

Introduction

Natural products have always contributed to the discovery and development of novel molecules. An analysis of all substances approved by the FDA as new molecular entities demonstrates that more than one-third of the total number of substances corresponds to natural products and their semi-synthetic derivatives (Patridge et al., 2015). Among natural products, plants are a rich source of therapeutic agents and bases for synthetic drugs. Despite the advances in organic synthesis, currently 25% of prescribed drugs worldwide are still derived from plant sources, thus showing that plant species are still an important source of novel drugs for diseases that continue to lack treatment option (Newman and Cragg, 2012; Rates, 2001).

The Eriocaulaceae family distributed in the mountainous regions of South America, especially in the rocky savannas of Brazil, contains about 1400 species belonging to eleven genera (Alvarado et al., 2013). *Paepalanthus*, with about 500 species, is one of its principal genera commonly found in the States of Bahia and Minas Gerais, Brazil. A number of studies have demonstrated that almost all species of *Paepalanthus* subgenus *Platycaulon* possess flavonoids, naphthoquinones, caffeic acid, and naphthopyranone derivatives, including paepalantine (**1**) and 8,8-paepalantine dimer isolated from *P. bromelioides* (Vilegas et al., 1990; Coelho et al., 2000), planifolin isolated from *P. planifolius* (Santos and Vilegas, 2001; Varanda et al., 2006), and 5-methoxy-3,4-dehydroxanthomegnin isolated from *P. latipes* Silveira (Kitagawa et al., 2004).

Currently, some of these compounds are of notable pharmacological interest owing to their proven biological activity; for example, it has been reported that the isocoumarin paepalantine (**1**), present in different extracts of *P. bromelioides* and *P. vellozioides*, exhibits a wide range of biological activities, including antimicrobial (Devienne and Raddi, 2002; Devienne et al., 2005), anti-inflammatory (Di Stasi et al., 2004), antioxidant (Kitagawa et al., 2003; Devienne et al., 2007), cytotoxic (Varanda et al., 1997; Devienne et al., 2002), genotoxic (Tavares et al., 1999) and

* Corresponding author.
E-mail: rodrigo.kitagawa@ufes.br (R.R. Kitagawa).

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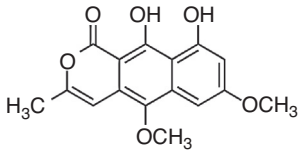
mutagenic activities (Varanda et al., 2004).



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Despite several studies reporting biological activities of paepalantine (**1**), the data regarding the behaviour of paepalantine in different solvents are limited. Studies performed with paepalantine often use dimethylsulfoxide (DMSO) as the solvent. However, this solvent is toxic to humans and experimental animal models (White et al., 2013; Galvão et al., 2014), induces apoptosis and disruption of the membrane in cell culture models (Ménorval et al., 2012; Yuan et al., 2014), interferes with antioxidant activity (Kabeya et al., 2013), and exerts multiple harmful systemic effects, thus making it a controversial molecule for research (Santos et al., 2003; Qi et al., 2008; Galvão et al., 2014).

The evaluation of its bacteriostatic potential *in vitro* showed that DMSO solution 30% is highly bactericidal towards a wide range of microorganisms (Jacob and Herschler, 1986). Thereby, DMSO in solution form interferes with the tests evaluating the bactericidal potential of natural products.

An important step in the development of a novel drug is the evaluation of its physicochemical characteristics and possible interactions with adjuvants that may be used for its development. Chemical interactions of the drug with potential solvents can alter its solubility, bioavailability, validity, and ultimately mask the assessed biological activity (Wang et al., 2006). Additionally, this step is important for determining the non-toxic adjuvants that can be used in animals and humans, while analytical methods for quality control of the molecule are investigated and evaluated (Gowthamarajan and Singh, 2010).

Taken together, the objective of the current study was to evaluate solvents that solubilise paepalantine, without any interference with its analysis and which have minimal toxicity in case of *in vivo* tests.

Material and methods

Plant material

Paepalanthus bromelioides Silveira, Eriocaulaceae, was collected in Serra do Cipo, State Minas Gerais, Brazil, and identified by Dr. Paulo Takeo Sano associated with the Institute of Biosciences, University of São Paulo. The voucher specimen (CFSC, 13839) was deposited at the Herbarium in the Department of Botany, Institute of Biosciences, University of São Paulo, Brazil.

Chemicals

Dimethyl sulfoxide (DMSO), nitrotetrazolium blue chloride (NBT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nicotinamide adenine dinucleotide (NADH), potassium persulfate, phenazine methosulfate (PMS), sodium borohydride, Trolox, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propylene glycol was purchased from Neon Comercial Ltda. (São Paulo, SP, BR). Paepalantine was obtained according to the procedure previously reported by Vilegas et al. (1990), and the stock solution was prepared at 10 mg/ml in different solvents including DMSO. Maximum absorbance of paepalantine was observed at 265 nm.

Solubility test

The solvents selected had values close to the dielectric constant of DMSO. Thus, propylene glycol, isopropanol, and glycerin were selected and evaluated in context of the dissolution profile of paepalantine taking the following parameters into consideration: concentration (10, 20 and 40 mg/ml), temperature (ambient or 37 °C), pH (5.0 and 7.5) and addition of surfactant (0.02% Tween 80 or Tween 20). The evaluation consisted of determination of homogeneity and transparency of the solution by visual analysis. The presence of paepalantine crystals was evaluated by optical microscopy (Olympus AX70, Olympus optical Co., Tokyo, Japan) using the appropriate software (Leica IM 1000 Software, Wetzlar, Germany). The microscope was equipped with a digital camera (Axiocam ERc 5s, Zeiss, Jena, Germany). Based on findings from the solubility tests, appropriate solvents were selected for future experiments.

UV spectrophotometry

Spectroscopy measurements were carried out by a UV/VIS spectrophotometer NANO DROP 2000 Thermo Scientific (Wilmington, USA). UV spectra at 200–800 nm were recorded for peak identification.

Validation of spectrophotometric analytical method

The items validation followed RE N°899 of May 29, 2003 of the Guide of Validation of analytical and bioanalytical methods from National Agency for Sanitary Vigilance (Anvisa, 2003).

Selectivity

The selectivity was determined from the comparison of the UV spectra of the following solutions: propylene glycol, DMSO, and paepalantine solutions in propylene glycol or DMSO.

Linearity

Paepalantine (**1**) (10 mg/ml) was dissolved in 1 ml of solvent (propylene glycol or DMSO). Aliquots of this solution were diluted in different concentrations: 0.1, 0.25, 0.37, 0.5, and 0.75 mg/ml, prepared in triplicate. The amount of paepalantine was determined by means of UV spectrophotometry. Calibration curves of concentration *versus* area were plotted, and the obtained data were subjected to regression analysis using the least-squares method.

Precision

Repeatability of spectrophotometric method was tested by analyzing concentrations levels of 0.1, 0.5 and, 0.75 mg/ml on three different days ($n = 10$). Paepalantine concentration was determined and the Relative Standard Deviation (RSD) was calculated.

Stability of solutions

Paepalantine (0.1, 0.25, 0.37, 0.5, and 0.75 mg/ml) stability in propylene glycol or in DMSO was determined by spectrophotometer. Post-preparation of the solutions analyses were carried out on days 1, 2, 3, 7, 14, 21, and 28 ($n = 10$) for every solution. Absorbance data were graphically represented as a function of time.

Antioxidant assays

Different strengths of the solvents were evaluated in varying concentrations of 0.31, 0.62, 1.25, 2.5, 5, 10% v/v. The tested paepalantine concentrations were 3.125, 6.25, 12.5, 25, 50, and

100 μg/ml. Trolox® was used as the standard antioxidant substance. The assays were performed in triplicate and repeated at least three times.

The results are expressed as percent inhibition (%Δ) and 50% effective concentration (EC_{50}). The equation used for calculating the percent inhibition was as follows: $\% \Delta = (A_0 - A/A_0)^*100$, where A_0 is the absorbance of the test solution without a sample (control), and A is the absorbance observed with sample.

ABTS$^{\bullet+}$ assay

The antioxidant activity against ABTS$^{\bullet+}$ was determined using a modified method originally described by Re et al. (1999). Initially, an aqueous mixture of ABTS (7 mM) and potassium persulfate (2.45 mM) was incubated at room temperature in the dark for 16 h. The solution formed from ABTS$^{\bullet+}$ exposure was diluted in ethanol to generate an absorbance of 0.7 at 734 nm. In a 96-well microplate, 300 μl of ABTS$^{\bullet+}$ and 3 μl of the samples at different concentrations were added. After 5 min, spectrophotometric measurements were carried at 734 nm using iMark® Microplate Absorbance Reader, from Bio Rad Laboratories (Washington, U.S.A.).

NBT assay

A modified superoxide radical test ($O_2^{\bullet-}$) was performed based on a method previously described by Suzumura et al. (1999). In a 96-well microplate, varying concentrations of paepalantine, phenazine methosulfate (PMS) (0.5 mM), nitrotetrazolium blue chloride (NBT) (0.045 mM) and phosphate buffer, pH 7.4, were added. The mixture was incubated for 2 min, followed by addition of 0.125 mM nicotinamide adenine dinucleotide (NADH). After incubation for 10 min at room temperature, spectrophotometric measurements were carried out at 560 nm.

Reaction with hypochlorous acid (HOCl)

HOCl reactions were studied using a previously described method by Ching et al. (1994), which was based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB). TNB was obtained by reducing a 1 mM solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in a 50 mM KH_2PO_4–KOH buffer (pH 6.6), containing 5 mM EDTA, and 20 mM sodium borohydride. In order to perform the assay, 25 mM HOCl was incubated with 50 mM TNB for 5 min in the presence or absence of paepalantine in a final volume of 200 μl. The ability of paepalantine to scavenge HOCl was determined by measuring the oxidation of TNB to DTNB at 412 nm, upon pre-incubating of paepalantine with HOCl, followed by addition of TNB.

Statistical analysis

The parameters were expressed as mean ± standard deviation. Data were analysed using analysis of variance (ANOVA) and linear regression analysis. Differences between groups were considered significant at $p < 0.05$.

Results and discussion

Preformulation studies assist scientists in screening lead candidates based on their physicochemical and biopharmaceutical properties. Preformulation information (permeability, solubility, and stability) is useful for selection of new chemical entities for preclinical efficacy/toxicity studies, which is a major section under investigational new drug applications (Bharate and Vishwakarma, 2013).

In this context, the present study evaluated the solubility of paepalantine in solvents other than DMSO, and the solvent's potential to interfere with antioxidant assays. According to results from the solubility test, paepalantine was soluble only in propylene glycol at 10 mg/ml, pH 7.5, with complete dissolution (no crystals were observed) as confirmed by optical microscopy analysis. Further, the use of surfactants was not favourable to solubilisation of paepalantine in propylene glycol and other selected solvents.

After the analysis of spectra of pure solvents and paepalantine's solutions, the analytical wavelength of choice was at 391 nm (Fig. 1).

Linear correlation was found for both propylene glycol and DMSO solutions containing paepalantine. The regression analysis data are shown in Fig. 2. The regression coefficient (R^2) obtained was higher than 0.98 which attests to the linearity of the method.

The repeatability of spectrophotometric method for the paepalantine's solutions (propylene glycol and DMSO) resulted in RSD values below 5% indicating the precision of the method.

The stability of paepalantine in propylene glycol and DMSO was assessed by spectrophotometric analysis in different concentrations during 28 days (Fig. 3). Paepalantine's solution in DMSO was considered stable during the period of storage, while paepalatine's solution in propylene glycol showed a concentration reduction ($p < 0.05$).

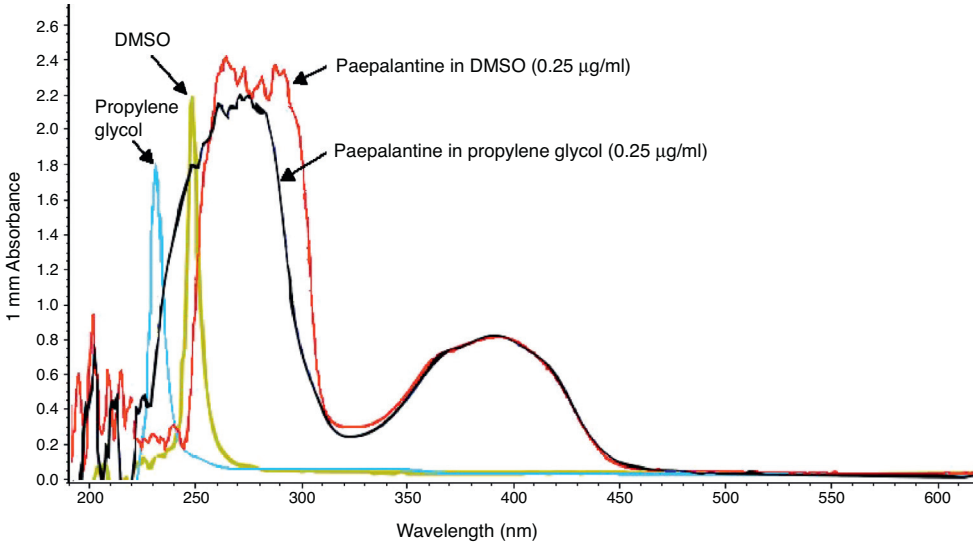

Fig. 1. Paepalantine absorption profile (200 at 600 nm).

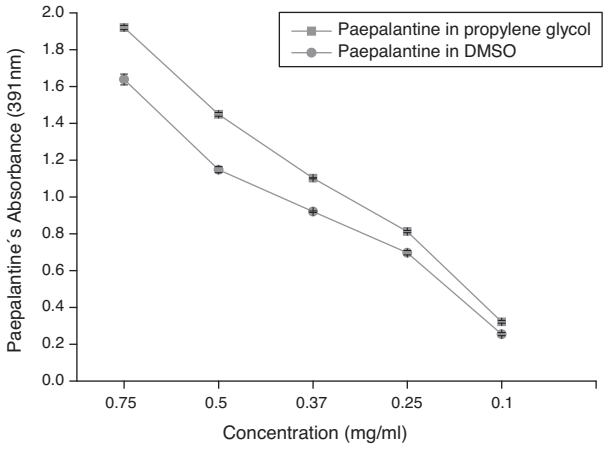

Fig. 2. Linearity analysis for spectrophotometric calibration curves. Paepalantine in propylene glycol ($R^2 = 0.99$; $a = -0.20$; $b = 1.33$) and Paepalantine in DMSO ($R^2 = 0.98$; $a = -0.17$; $b = 1.58$). All absorbances were measured in triplicate.

Despite DMSO, being widely used as a universal solvent for natural products especially plant-derived products, previous studies have reported interference from this solvent owing to its toxic effects on various cell types, microorganisms, and animal models. Hence, it has been replaced with other suitable solvents for in *in vivo* experiments (Basch and Gadebusch, 1968; Vogin et al., 1970; Qi et al., 2008; Hanslick et al., 2009; Ménorval et al., 2012; White et al., 2013; Yuan et al., 2014). Propylene glycol is a frequently co-administered solvent in formulations used in preclinical and clinical studies. This solvent is generally nontoxic and noncarcinogenic, and commonly used solvent for oral, intravenous, and topical pharmaceutical agents (Kulo et al., 2012; Fiume et al., 2012; Healing et al., 2015).

Some studies have reported the ability of DMSO to act as a free radical scavenger, which gives it antioxidant properties. The antioxidant properties of DMSO limit its use as a solvent for the development of new antioxidant drugs (Kabeya et al., 2013; Sanmartín-Suárez et al., 2011; Bektasoglu et al., 2006).

Coumarins are low-molecular weight phenolic compounds and have various biological properties, including antioxidant activity. In general, free radical scavenging and antioxidant activity of this class of compounds mainly depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring (Kostova et al., 2011).

Table 1
Effective concentration (EC_{50}) of paepalantine dissolved in propylene glycol and DMSO and Trolox in $ABTS^{\bullet+}$ assay.

Compound	EC_{50} (μg/ml)
Paepalantine in propylene glycol	2.42 ± 0.5
Paepalantine in DMSO	3.33 ± 0.56
Trolox	1.07 ± 0.04

Previous studies have reported the antioxidant potential of paepalantine in the respiratory burst of neutrophils (Kitagawa et al., 2003) and in the mitochondria (Devienne et al., 2007). In this study, we evaluated the scavenging capacity of this compound against the ABTS radical, reactive oxygen species such as $O_2^{\bullet-}$, HOCl, and the interference from solvents, DMSO and propylene glycol, in these assays. The effect of solvents on $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ assays is shown in Fig. 4.

According to these data, DMSO and propylene glycol do not interfere with these assays and the antioxidant effect of paepalantine is unchanged, which is similar to Trolox (Fig. 5 and Table 1).

However, DMSO had an antioxidant effect against HOCl at all concentrations tested. On the contrary, this phenomenon was not observed with propylene glycol, which shows interference only at higher concentrations that were not employed in the test with paepalantine (Fig. 6). Kabeya et al. (2013) observed the antioxidant interference of DMSO in assays involving capture of HOCl. Studies suggest that the presence of the sulphur atom in the molecule favours the oxidation of DMSO by HOCl (Peskin and Winterbourn, 2001; Ruff et al., 2012), directly influencing the results.

Paepalantine dissolved in propylene glycol showed higher antioxidant activity in this assay, which seemed to be better than Trolox (Fig. 7).

The results showed that paepalantine has significant antioxidant activity against the reactive species studied, similar to or better than that observed with Trolox. Further, paepalantine possesses 9-OH and 10-OH groups providing a catechol-like arrangement as well as a planar structure, both of which are highly favourable to electron delocalization that is similar to the orthodiphenolic arrangement on the B-ring of flavonoids, which is a well-established requirement for the ROS-scavenging activities of polyphenols (Pietta, 2000; Tejero et al., 2007; Devienne et al., 2007).

The results of the current study demonstrate that propylene glycol can be an alternative to the use of DMSO which shows interference in biological assays as antioxidant assays.

Additionally, previous studies showed that paepalantine is a promising compound for pharmaceutical use; therefore this study

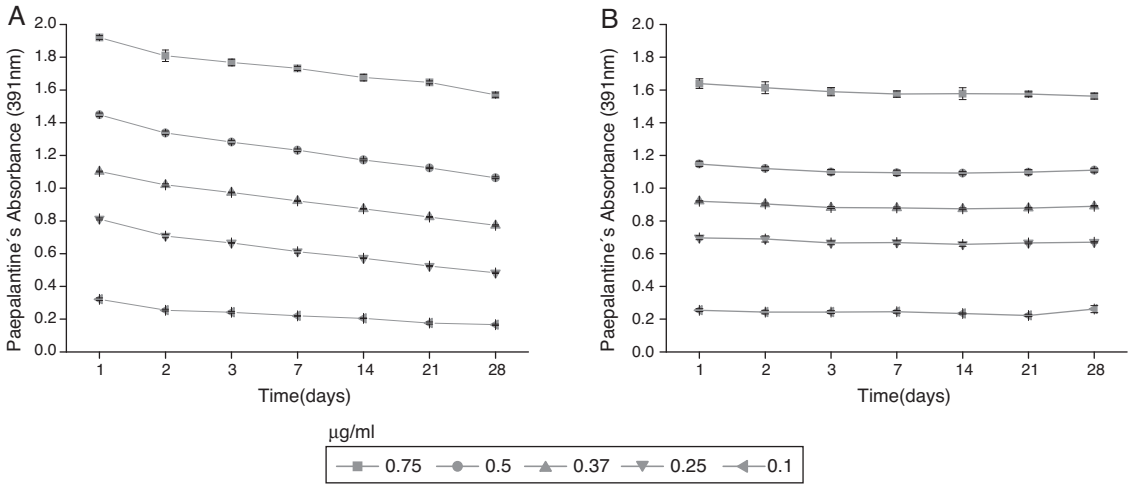

Fig. 3. Paepalantine absorption over storage. (A) propylene glycol; (B) DMSO. Values are expressed as mean ± standard deviation ($n = 3$).

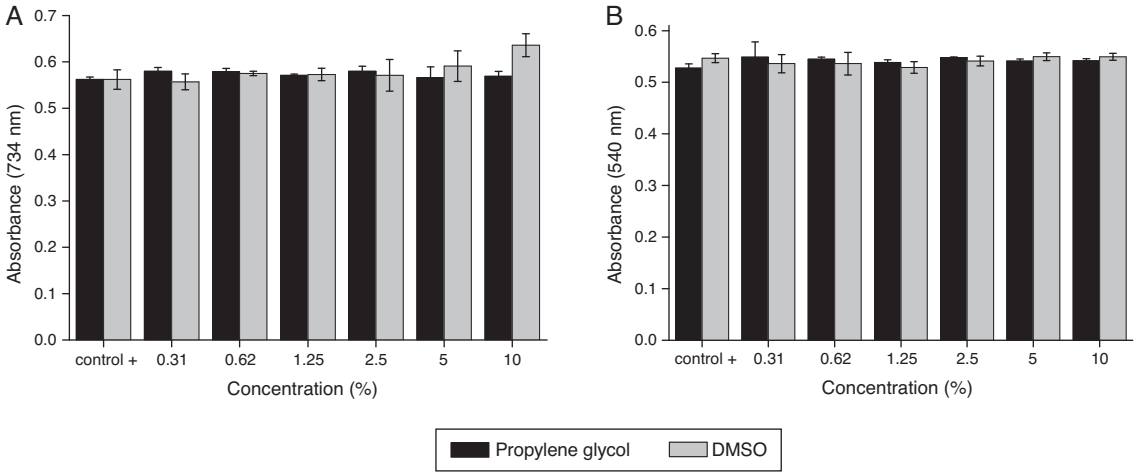

Fig. 4. Scavenging effect of propylene glycol and DMSO (v/v%) against $ABTS^{\bullet+}$ (A) and $O_2^{\bullet-}$ (B).

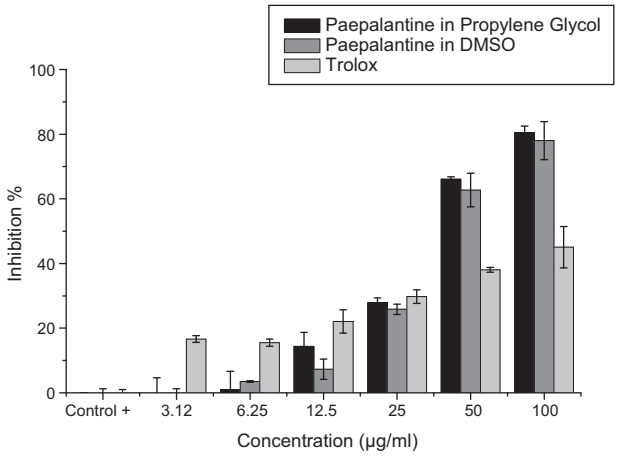

Fig. 5. Antioxidant activity of paepalantine in propylene glycol and DMSO and Trolox in the NBT assay ($O_2^{\bullet-}$).

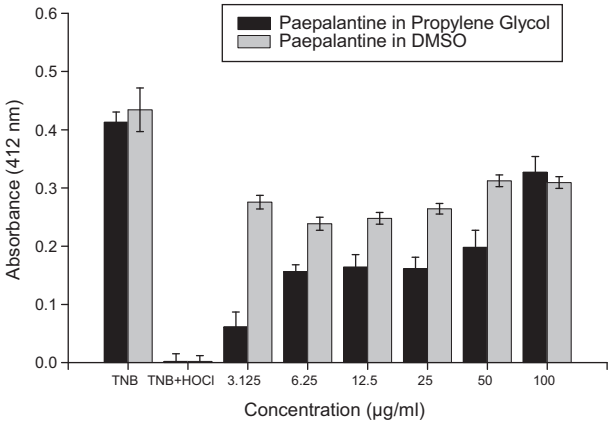

Fig. 7. Antioxidant activity of paepalantine dissolved in propylene glycol and DMSO and Trolox in the TNB assay (HOCl).

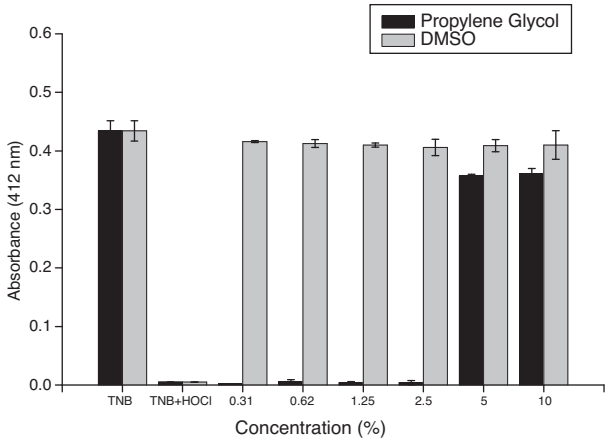

Fig. 6. Effect of propylene glycol and DMSO (v/v%) on HOCl scavenging.

may lead to future development of a formulation with solvent compatible for preclinical and clinical studies and more appropriate under the technological view.

Authors' contributions

JPLD contributed to running the laboratory work for isolation and identification of the paepalantine, biological studies, analysis of the data, and drafting the paper. RRK designed the study, supervised the laboratory work for the isolation and identification of the paepalantine and antioxidant test, and contributed to critical reading of the manuscript. CS supervised the laboratory work in the preformulation studies and contributed to critical reading of the manuscript. RCRG supervised the laboratory work in antioxidant test and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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