Study of the *in vitro* release profile of sesquiterpenes from a vaginal cream containing *Copaifera duckei* Dwyer (Fabaceae) oleoresin

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ABSTRACT

Copaiba trees and their oleoresin is an important natural product used as an anti-inflammatory and antimicrobial drug, in particular for gynecological infections. The *in vitro* release profile of sesquiterpenes in a vaginal cream containing copaiba (CVC – copaiba vaginal cream) oleoresin in buffered solutions at different pHs was evaluated in the present study. The dissolution test revealed that *trans*-caryophyllene (TC) was constantly released until the final test time. The maximum peaks were found at a concentration of 478.79 nL/mL for the buffer at pH 5 and at a concentration of 475.99 nL/mL for the buffer at pH 6, with a percentage release of 50.52% and 50.21%, respectively. Likewise, the caryophyllene oxide (CO) was released into the buffers at pHs 5 and 6, with maximum release peaks at 60 minutes at a percentage of 31.8% and 33.5% corresponding to concentrations of 2.779 and 2.925 μg/mL, respectively. The release and dissolution profiles of the sesquiterpene compounds indicated that a pH of 5-6 in the dissolution media is adequate for release of these markers of antimicrobial activity in *Copaifera duckei* Dwyer. Therefore, these conditions possibly favor the therapeutic action of CVC.

INTRODUCTION

Copaiba trees belong to the genus C*opaifera* L.*,* family Fabaceae, subfamily Caesalpinioideae. These plants are slow-growing trees, measuring 25-40 m in height, featuring a rough and dark trunk 0.4-4 m in diameter and living up to 400 years of age (Silvia *et al*., 2008). Copaiba trees are native to the tropical regions of Latin America and Western Africa and can be found in the Americas in the region spanning from Mexico up to the north of Argentina (Veiga-Júnior and Pinto, 2002). The genus *Copaifera* comprises 72 species, 16 of which can only be found in Brazil (Dwyer, 1951); the species *Copaifera duckei* Dwyer is prominent among them. The copaiba oleoresin is an important natural product used in folk medicine as an anti-inflammatory, anti-septic, and wound healing substance, especially for the upper airways and urinary tract (Basile, 1988; Carvalho and Cascon, 2003; Carvalho, 2005). Some authors report that the oleoresin is a detoxification product of the plant organism, functioning as a defense mechanism against animals, fungi, and bacteria (Alencar, 1982; Macedo and Langenheim, 1989). Various pharmacological studies of the copaiba oleoresin have already been described in the literature, including the compound's antimicrobial and antibacterial (Opdyke, 1976; Miranda *et al*., 2000; Tincusi *et al*., 2002), analgesic (Carvalho *et al*., 2005), anti-inflammatory (Fernandes *et al*., 1992; Carvalho et al, 2005), wound healing (Brito, 1999), gastroprotective (Paiva *et al*., 1998), and antitumoral (Ohsaki *et al*., 1994; Lima *et al*., 2003) activities. Antimicrobial studies have revealed that the oleoresins of various copaiba tree species exhibit this activity, which may be related to the terpenic compounds present in the oleoresin (Muroi and Kobo, 1993; Sharfin, 2002; Santos *et al*., 2008). Studies conducted by Santos *et al*. (2008) using electron microscopy corroborate this hypothesis because *Staphylococcus aureus* strains underwent changes, such as the loss of their cell walls and the release of cytoplasmic compounds, after treatment with oleoresin.

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Lima *et al*. (2011) demonstrated that a vaginal cream containing 2.5% copaiba oleoresin is safe during gestation in female rats (*Rattus norvegicus*) of the Wistar strain.

The normal physiological pH in the vagina varies between 3.8 and 4.2. These acidic values result from the secretion of organic acids by the vaginal epithelial cells and from the production of lactic acid from glycogen by bacilli and lactobacilli constituting the normal vaginal microbiota (Jarmy-Di Bella *et al*., 2009).

Among the most frequent vaginal infectious processes, bacterial vaginosis, vulvovaginitis, and trichomoniasis are the most prominent (Sobel, 1990). Several of these polymicrobial infections promote changes in the microbiota, significantly reducing the number of lactobacilli and increasing the pH to values above 3.5, which favors the growth of bacteria such as *Gardnerella vaginalis, Mycoplasma hominis, Mobiluscus* sp*.,* and *Bacteroides* (Murta *et al*., 2000; Adad *et al*., 2001; Marrazzo, 2003).

Extremely acidic vaginal pHs are commonly found in fungal infections, which are generally caused by *Candida* sp. Conversely, alkaline vaginal pH values are mostly associated with *Trichomonas vaginalis* infections and with bacterial vaginosis caused by *Gardnerella vaginalis*. Depending on factors such as age, vaginal microbiota, and menstrual period, the vaginal pH can range between 3.8 and 7 (Jarmy-Di Bella *et al*., 2009).

The aim of the present study was to evaluate the *in vitro* release profile of sesquiterpenes from a vaginal cream containing 2.5% copaiba oleoresin in buffered solutions at pHs ranging from 3 to 6, which are the threshold pH values usually found in vaginal fluids exhibiting a modified microbiota.

MATERIALS AND METHODS

Obtainment of the oleoresin and copaiba vaginal cream (CVC)

The oleoresin used for analysis and pharmaceutical development was obtained from the company Beraca Ltd., located at Ananindeua, in Pará State, Brazil. According to the company's report, the oleoresin belongs to the species *Copaifera duckei* Dwyer.

The CVC and the base cream were obtained at a concentration of 2.5%, supplied in polyethylene bottles, from the Pharmaceutical Laboratory Almeida Prado, Ltd., in the city and state of São Paulo, Brazil.

CVC dissolution assay

Buffer solutions were prepared at pH 3, 4, 5, and 6, using hydrochloric acid (HCl) and 0.2 M sodium hydroxide (NaOH) in distilled water (Farmacopeia Brasileira, 2010). One hundred milliliters of each buffer solution was placed in dissolution tanks of the dissolution test system model 299 (Nova Ética, Ltd. São Paulo, Brazil) with the paddle apparatus, a stirring rate of 40 RPM, and a controlled temperature of $37 \pm 1°C$. Exactly 5 g of CVC at a

concentration of 2.5% oleoresin was used for the release profile analysis. The dissolution and release profiles were obtained from the quantification of the levels of phytochemical sesquiterpenoid compounds, caryophyllene oxide (CO) ($C_{15}H_{24}O$), and *trans*-caryophyllene (TC) ($C_{15}H_{24}$) released from the CVC into the solutions in the tanks at the timepoints of 10, 30, 60, 90, 120, and 150 minutes.

One-milliliter aliquots were collected at the given time points for the extraction of the non-polar fraction by liquid-liquid partitioning with 1 mL of hexane. After separation, the hexane fraction was analyzed by gas chromatography.

Analysis of the samples obtained in the dissolution test

The analysis of the samples was conducted by gas chromatography using the method described by Lima *et al*. (2011). The sesquiterpene contents were calculated from the equation of the standard curve obtained using CO (Sigma-Aldrich, lot 128H3429) at concentrations of 5, 10, 20, 40, and 80 μg/mL and TC (Sigma-Aldrich, lot BCBJ3860V) at concentrations of 250, 500, 1,000, 2,000, and 4,000 nL/mL, using the average of three measurements (Figures 1, 2, 3, 4, 5, 6 e 7).
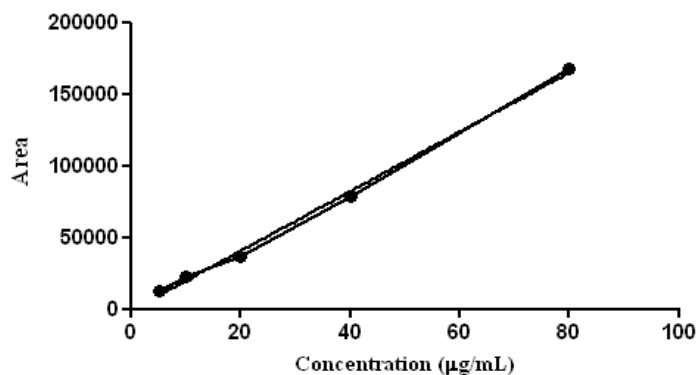

Fig. 1: Standard CO curve obtained using GC-FID at concentrations of 5 to 80 μg/mL, with the equation $y = 1862x - 4.208$ and the coefficient of determination $R^2 = 0.9969$.
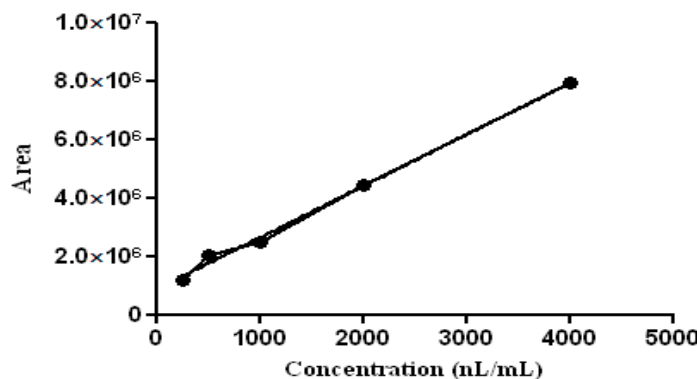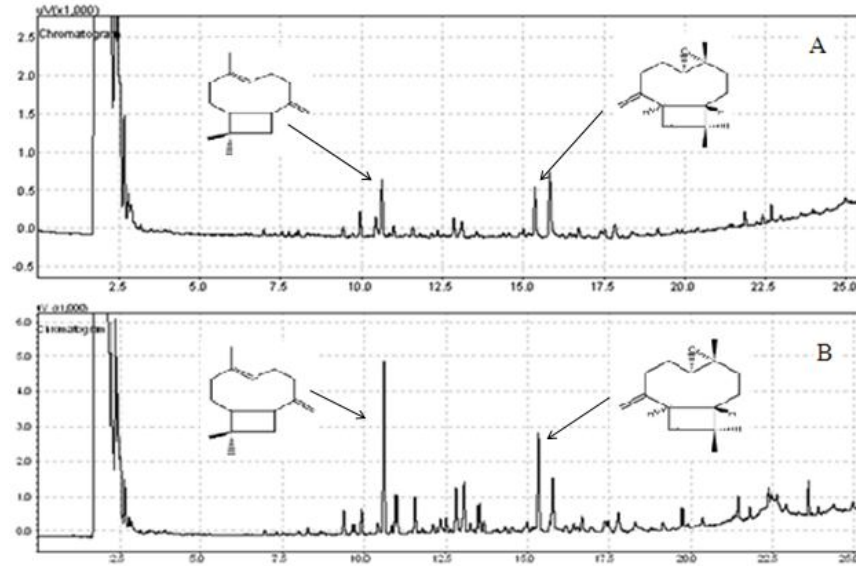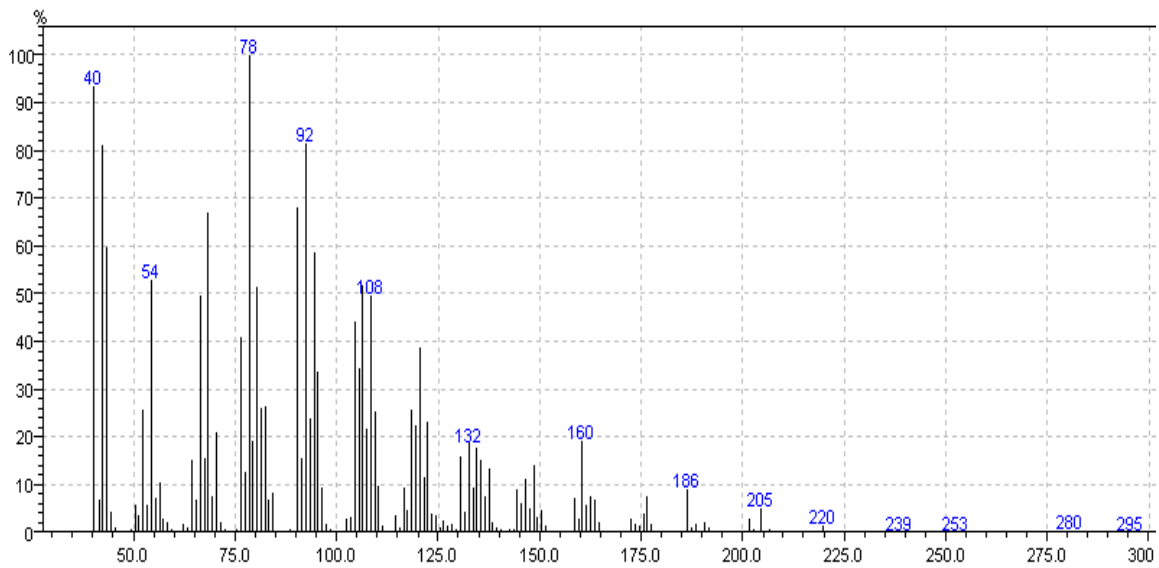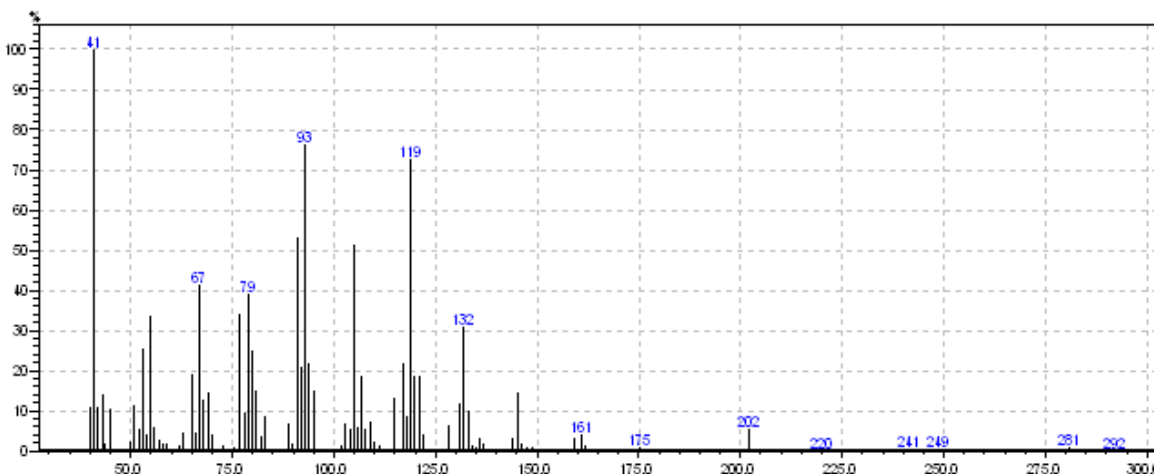

Fig. 2: Standard TC curve obtained using GC-FID at concentrations of 250 to 4,000 nL/mL, with the equation $y = 1555x - 821.1$ and the coefficient of determination $R^2 = 0.9959$.

Fig. 3: Comparison of chromatographic profiles of CVC (A) and *C. duckei* oleoresin (B) indicating the quantified sesquiterpenes (CO and TC), obtained by GC-MS with the injection of 1 μL in the split mode (20:1 split ratio).

Fig. 4: Fragmentation profile of the sesquiterpene standard CO ($C_{15}H_{24}O$, M 220), obtained by GC-MS (QP2010SE).

Fig. 5: Fragmentation profile of the sesquiterpene CO ($C_{15}H_{24}O$, M 220) of the copaiba oil (*Copaifera duckei* Dwyer), obtained by GC-MS (QP2010SE).

Fig. 6: Fragmentation profile of the sesquiterpene standard TC ($C_{15}H_{24}$, M 204), obtained by GC-MS (QP2010SE).

Fig. 7: Fragmentation profile of the sesquiterpene TC ($C_{15}H_{24}$, M 204) of copaiba oil (*Copaifera duckei* Dwyer), obtained by GC-MS (QP2010SE).

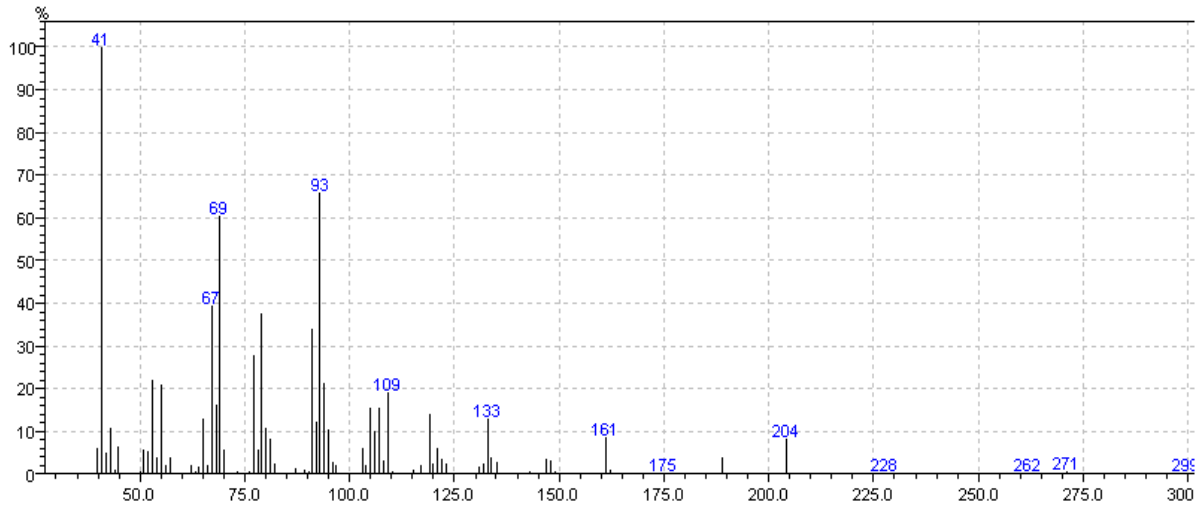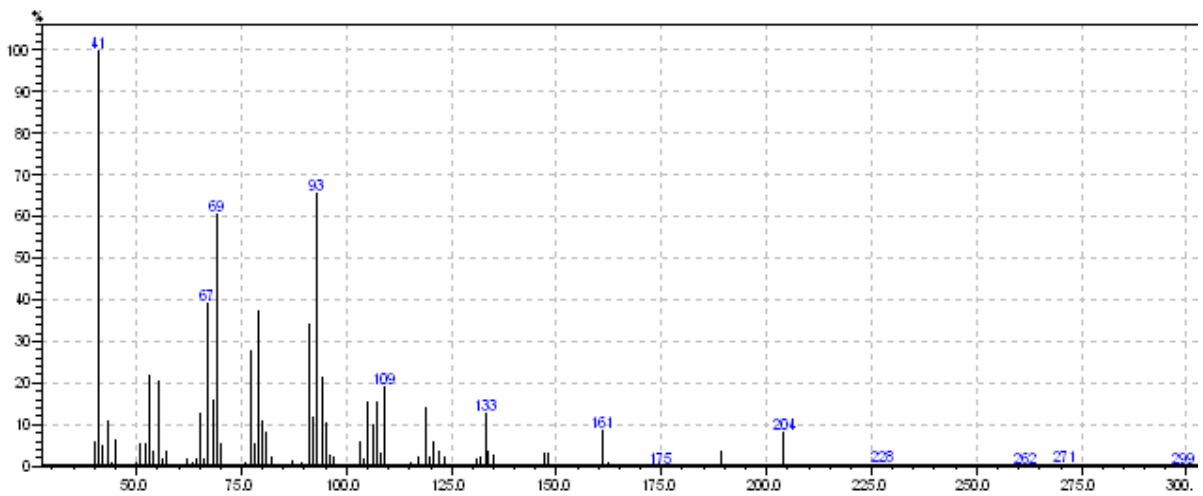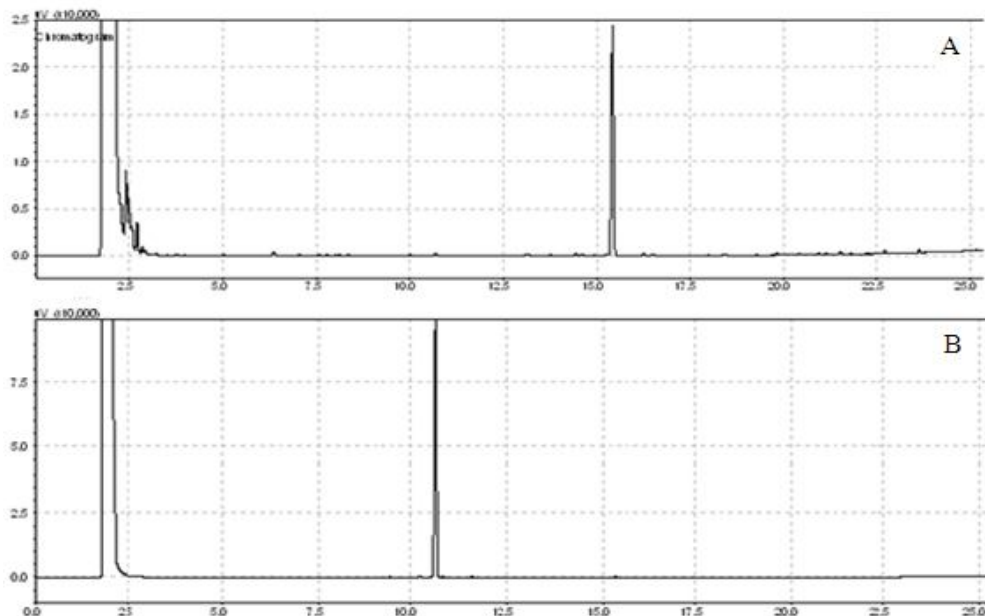**Fig. 8:** Comparison of chromatographic profiles of standard compounds, where (A) CO and (B) TC were obtained by GC-MS with retention times of 15.38 and 10.64 minutes, respectively.

A gas chromatograph (Shimadzu GC-plus 2010) with a flame ionization detector (GC-FID) was used with a capillary column Rtx-1 consisting of polydimethylsiloxane (30 m x 0.25 mm ID x 0.25 μm) as the stationary phase. Helium was used as the carrier gas at a flow of 1.5 mL/minute. The oven temperature was programmed as follows: the temperature was maintained at 120°C for 2 minutes, then increased at a rate of 3°C/minute up to 160ºC, which was maintained for another 2 minutes, and finally increased at a rate of 8ºC/minute up to a final temperature of 290ºC, which was maintained for 5 minutes. The injector and detector temperatures were maintained at 270°C and 290°C, respectively. A volume of 1.0 μL (hexane fraction) was injected using a split ratio of 20:1. A gas chromatograph coupled to a mass spectrometer (QP2010SE) (GC-MS) was used under similar conditions to the GC-FID, operated in the Scan 40-400 (*m/z*) mode, with an electron impact of -70 eV.

Statistical analysis

The results from each analysis were statistically analyzed using ANOVA followed by a linear regression test. The dissolution profiles were compared using the Student-Newman-Keuls test, and results with p values less than 0.05 were considered significant. The GraphPad Instat® and Prism® version 5.03 software were used.

RESULTS AND DISCUSSION

The analysis of the copaiba oleoresin by gas chromatography with a flame ionization detector was undertaken using external standards with a CO content of 7.746 ± 0.022 μg/mg (n = 3), which corresponded to a percentage area of 2.68 ± 0.19%, and using a TC content of 946.67 ± 14.34 nL/mL (n = 3), with an area of 9.34 ± 0.34%. The retention times of both compounds were 15.38 and 10.64 minutes, respectively (Fig. 8). The GC-MS analysis of non-esterified *C. duckei* oleoresin samples undertaken by Lima (2011), using the same chromatographic conditions with the only exception of the chromatographic column, also revealed the presence of CO and TC in the species. However, the retention times were 9.39 and 6.01 minutes. TC and its corresponding oxide are sesquiterpenes usually found in the various copaiba species and in many essential oils of a number of species from different botanic families. These two compounds may actually represent the major part of sesquiterpenes present in the species as these compounds yield numerous pharmacological effects, including anti-inflammatory (Shimizu, 1990), antitumoral (Zheng *et al*., 1992)**,** spasmolytic (Duke, 1992), and antimicrobial activities that protect the plant by inhibiting fungal and bacterial proliferation (Kang *et al*., 1992, Veiga-junior, 2002). The *in vitro* dissolution test is critical for assessing the drug performance and for correlation with *in vivo* results (Amidon *et al*., 1995). The active principle must be completely solubilized, with total disintegration of its carrier, being constituted solely by small particles capable of making contact with the biological membranes so that the drug can be absorbed (Ansel *et al*., 2000). Thus,

controlling the experimental conditions is crucial to guaranteeing the reliability and reproducibility of the test. The CVC dissolution test using TC as the marker displayed a progressive release until the final dissolution timepoint, with maximum concentration peaks of 478.79 nL/mL for the buffer at pH 5 and 475.99 nL/mL for the buffer at pH 6 and with a percentage release of 50.52% and 50.21%, respectively. The dissolution media at pHs 5 and 6 exhibited a significant difference relative to the other dissolution media (Fig. 9). For the CO marker, the release was more efficient at pHs 5 and 6, with maximum peaks at 60 minutes and a higher statistical significance compared with the other dissolution media. The CO-release percentages were 31.8% and 33.5%, corresponding to concentrations of 2.779 μg/mL and 2.925 μg/mL, respectively. Poor dissolution occurred in the buffers at pHs 3 and 4. A maximum CO release value of 2.059 μg/mL in 90 minutes was observed at pH 3 and was higher than the value obtained in the buffer at pH 4, which was 1.968 μg/mL in 60 minutes, equivalent to a percentage of 22.5%. The buffer solutions at pHs 5 and 6 effectively led to CVC dissolution. Although a higher CO release was obtained at pH 6, a better profile was observed at pH 5, exhibiting a gradual and uniform increase of the release during the course of the dissolution, up to 120 minutes (Fig. 10).
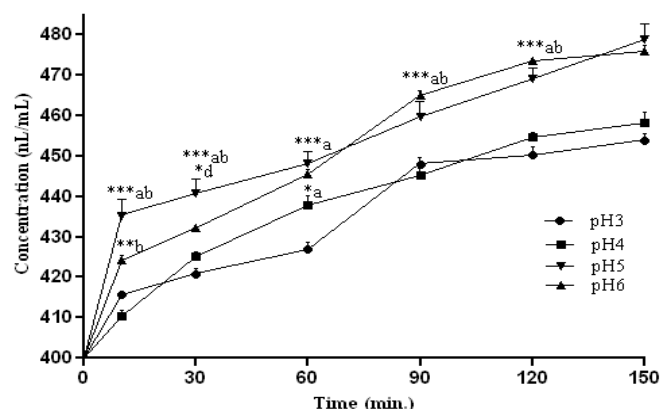
Fig. 9: Dissolution and release profiles of CVC TC in different buffer solutions. Student-Newman-Keuls test, where *p < 0.05, **p < 0.01, and ***p < 0.001; (a) comparison with pH = 3, (b) comparison with pH = 4, and (d) comparison with pH = 6.

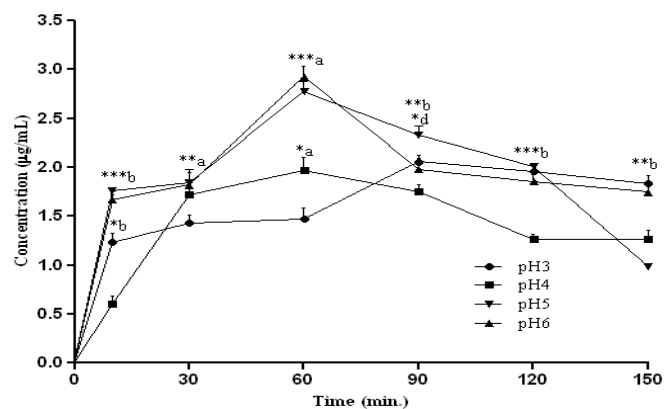**Fig. 10:** Dissolution and release profiles of CVC CO in different buffer solutions. Student-Newman-Keuls test, where *p < 0.05, **p < 0.01, and ***p < 0.001; (a) comparison with pH = 3, (b) comparison with pH = 4, and (d) comparison with pH = 6.

CONCLUSION

In conclusion, the CVC dissolution was tested in different buffered solutions that simulated the vaginal pH. The results demonstrated that better release of the phytochemical markers was observed at pHs 5 and 6. Taking into consideration the CVC dissolution and release profiles, this phytotherapeutic product is expected to exhibit greater therapeutic action at vaginal pH conditions ranging from 5 to 6.

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