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Design invitro characterization and optimization of suppositories of *Vernonia amygdalina* leaf extract for possible clinical trials in the treatment of vulvovaginal candidosis

*Omeh R. C.

Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Agbani, Enugu State, Nigeria

ABSTRACT

The antifungal properties of bitter leaf extracts have been widely reported. Full clinical use of this material in both orthodox and alternative medicines especially through the oral route is hampered by its extreme bitter taste and the absence of formal clinical trial studies required for establishing its effectiveness, safety and posology. The aim of the current work was to formulate bitter leaf extract as suppositories thereby enabling its administration through the vagina and therefore avoiding the bitter taste and low patient acceptability associated with the oral route delivery. The extract was obtained from the fresh leaves of the plant by hot maceration for 24 h in distilled water maintained at a temperature of 60 °C. The solution obtained was concentrated on a rotary evaporator to a sticky mass which was then oven-dried for 24 h at 60 °C. Phytochemical analysis, visible/ultraviolet light absorption and Fourier's transform infrared spectroscopies as well as acute toxicity studies were conducted on the extract. Varied weight proportions of the extract were incorporated into five selected suppository bases including cocoa butter, witepsol and three different grades of polyethylene glycol by the melt fusion technique. The formed suppositories were optimized for some physico-pharmaceutical and fungicidal activities. Parameters investigated, including hardness, pH, melting point and disintegration time were in the ranges of; 2.0 ± 0.84 - 3.1 ± 0.05 (kg/cm²), 5.95 – 6.89, 35.03 – 37.44 °C and 16.02 ± 0.35 - 27.45 ± 0.60 min respectively. All the formulations complied with official specifications for weight uniformity and active ingredient contents. The average dissolution rate of the various formulations was 2.325 ± 0.87 mg/min. Formulation SP8 which contained 40 % of the extract and 60 % PEG-4000 exhibited comparatively better formulation profiles and higher sustained release of extract and was, as such, adjudged as the optimized formulation. The formulation exhibited a 19.86 ± 0.37 mm zone of inhibition diameter on the *C. albican*-inoculated Sabouraud dextrose agar media. The current work concluded that aqueous leaf extracts of the *V amygdalina* plant possess antifungal properties and that intra-vaginal delivery of the extract via PEG-600 based suppository provided sustained release profile of the extract with potential for further clinical trials in the treatment of VVC..

Keywords: Suppository, Vulvo vaginal candidosis, *vernonia amygdalina*, extraction, phytoconstituents, antifungal activity.

*Corresponding author: Dr Romanus C. Omeh, Phone No: 07067175126, Email: romanus.omeh@esut.edu.ng & chijiokerom@gmail.com

Introduction

Bitter leaf (*Vernonia amygdalina*) is a multipurpose vegetable plant with both nutritional and ethnomedicinal uses [1, 2]. Its use in many cultures for therapeutic purposes dates

back to antiquity whereas recent research has reported that the leaf, bark and root extracts possess anti-inflammatory, antidiabetic, antibacterial, anthelmintic antimalarial and antifungal activities [3, 4].

Traditional oral administration methods of the bitter leaf plant materials include ingestion of the extract by chewing and swallowing of the raw leaves, use of the young stems as chewing stick, drinking of the aqueous extract obtained by hand squeezing of the fresh leaves in water as well as consumption of the cooked leaves as vegetable. Recent pharmaceutical research efforts have given rise to the formulation of powdered dry leaves of *V. amygdalina* as oral tablets and capsules. A major drawback to these attempts is the extreme bitter taste of the extract which most people including adults can hardly withstand. This challenge is of even greater concern with regards to oral administration of crude parts and uncoated tablets where direct contact with the taste buds is involved

A possible solution to these challenges is the formulation of the plant materials as dosage forms that bypass the oral cavity to deliver the extract directly to local sites or systemically. Rectal and vaginal suppositories are among dosage forms that can be used to achieve this option. Delivering bitter leaf extract as suppositories has the additional advantages of avoiding both the effects of digestive enzymes and the first pass metabolism in the stomach and liver as well as negative food effects and inconveniences associated with invasive routes of drug delivery. For the treatment of local vaginal infections like vulvovaginal candidosis, bacterial vaginosis and inflammations, local delivery of the extract may enhance higher local concentration and longer exposure to pathogens [3, 4].

Vulvovaginal candidosis (VVC) also medically known as vaginal candidiasis or candida vaginitis has been described as “a symptomatic vaginal infection especially caused by candida species” [5]. The infection may occur as acute or recurrent where the later has been defined as three or more episodes of symptomatic VVC occurring in less than one year [6]. It affects all women without racial, social, age or ethnic barriers [7, 8, 9]. Current disease prevalence reports show that about 75 % of all women experience at least one incidence of VVC during their lifetime and that about half of these (40 – 50 %) will experience at least one case of recurrent infection [10, 11]. Globally, 138 million women are reported to experience recurrent VVC annually and the average prevalence rate has been put at 3871 per 100,000

women [12]. Current drug treatment of VVC include, clotrimazole (72 %), fluconazole (14 %) and nystatin (6 %) [13]. Although these drugs have enjoyed good candida susceptibility, incidences of resistance have been reported and is rising in recent times especially among the recurrent infection cases [7]. Given the obvious heavy global burden of VVC, there is the need to expand both pharmacological and non-pharmacological measures for its management. Herbal remedies, due to their safety profiles, documented effectiveness and wide acceptance among the rural population [14] come handy as good supplement or alternative to the orthodox medications. Plant based extracts with suspected vaginal antifungal properties may show greater local effectiveness when administered as vaginal suppositories than through the oral route.

MATERIALS AND METHODS

Fresh leaves of *V. amygdalina* plant were purchased from Ogige market in Nsukka town of Enugu State, South-East Nigeria. Cocoa butter and propylene glycol were obtained from Loba Chemie (Mumbai, India) while three grades of polyethylene glycol (PEG-1000, PEG-4000 and PEG-6000) and tween 80 were products of BASF Inc. (Mumbai India). Witepsol H15 and Sabouraud agar were obtained from Sigma Aldrich. All other chemicals and reagents were purchased from a licenced vendor, Jechoem Chemicals limited, Nsukka, Enugu State, Nigeria and they were of analytical grades and used as supplied except otherwise specified.

Aqueous extraction of *V. amygdalina* leaves

The *V. amygdalina* leaves were identified by a Taxonomist, Mr Jude Ozioko of the Department of Plant Sciences, University of Nigeria Nsukka, Enugu State, Nigeria who also issued the voucher number of UNN/PLS/VS.043/2023 and retained a voucher specimen in the department's herbarium. The leaves were washed thoroughly under running tap water and then rinsed with distilled water. They were, thereafter, shade-dried for 10 days and pulverized to fine powder. About 8 g of the powder was macerated in 1.5 L of warm distilled water for 24 h. Thereafter, the extract solution was filtered through a cotton wool pack and then re-filtered through a No. 1(1001 110) Whatman filter paper The filtrate was then concentrated on a rotary evaporator (Hei-Vap Core, Heidolph Instruments, GMB

Germany) at a temperature of 75 ° C. The resulting residue was oven-dried for 4 h at a constant temperature of 60 ° C and thereafter placed in an amber glass bottle and stored in a refrigerator for further uses.

Qualitative phytochemical analysis of *V. amygdalina* extract

Various standard analytical procedures reported in Trease and Evans [15] for determining flavonoids, phenolic compounds, tannins, saponins, glycosides and alkaloids were used to investigate the phytochemical constituents present in the extract.

UV-VIS absorbance spectroscopy of extract solution

A 0.025 mg/ml solution of the extract in tween 80 was scanned through wavelength range of 200 to 600 nm on a UV-Vis spectrophotometer (JENWAY 7305, Germany) using the solvent as blank. The absorbance data obtained were plotted against corresponding wavelengths to produce an absorbance spectrum. In the next step, serial dilutions of the extract having concentrations of 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 (mg/ml) were prepared. A calibration curve of the extract was obtained by plotting the absorbances against the concentrations.

Oral acute toxicity studies of *V. amygdalina* leaf extract

Animal test was approved by the ethical committee of the Enugu State University of Science and Technology which issued a clearance certificate number ESUT/AEC/0205/AP129.

The method of Lorke [16] was employed to determine the oral acute toxicity of the extract. A total of 9 Wister rats of both sexes weighing 180 – 200 g were procured from the Department of Veterinary Pharmacology, University of Nigeria, Nsukka. The animals were housed in a laboratory cage and left to acclimatise with the environment for seven days prior to the tests while being allowed free access to food (Grower's mash rat pellet) and water. The study was carried out in 2 phases. In phase one, 3 randomly selected test animals designated as, 1, 2 and 3 received 10, 100 and 1000 (mg/kg body weight (mg/kg bw) of the extract respectively. and were thereafter observed for 24 h for behavioural changes, morbidity or death. In

stage two, another 3 groups of animals made up of one animal per group were given intragastrically 1600, 2900 and 5000 (mg/kg bw) of the extract and again observed for 24 h. Information obtained were used to calculate the LD₅₀ of the extract using equation 1. [16]

$$LD_{50} = \sqrt{(D_0 \times D_{100})} \text{ ----- (Eqn 1)}$$

where D₀ = highest dose of extract that caused no mortality and D₁₀₀ = lowest dose that caused death of any animal.

FTIR analysis of *V. amygdalina* extract and suppository formulation

The study was carried out on an FTIR equipment, (Perkin Elmer Model 1600, USA) using a thin film of the extract dispersion obtained by slow sonication at 1200 rpm. A smear of the dispersion was made on the chloride crystal cell of the equipment and then sandwiched with a second cell. A scanning range of 4000 to 400m⁻¹ was employed to obtain the spectra.

Determination of the displacement value of the extract

The volume of the cavity of a given suppository mould is fixed (constant) and so is the volume of the suppository formulation the cavity can hold. Introduction of an active ingredient into a base causes a displacement of its volume equivalence of the pure base. Displacement value of an active ingredient (in this case, the extract) is the amount of the extract that displaces one-unit part of the suppository base. This parameter is used to calculate the exact quantity of a given base needed to formulate suppository containing predetermined amount of an active ingredient. In the current work, the displacement values of the extract in respect of the selected five suppository bases were calculated using equation 2 and values obtained used in determining the quantity of each base used for specific formulations.

$$F = \left[\frac{100(E-C)}{GX} \right] + 1 \text{ ----- (Eqn 2)}$$

where,

F = displacement value, E = weight of blank (without extract) suppository base, G = weight of extract-loaded suppository while X = extract content in percentage.

Formulation of *V. amygdalina* extract vaginal suppository

Materials for the suppositories were selected based on documented characteristics with respect to expected quality attributes of the final product [17]. PEG 1000, PEG-4000 and PEG-6000, Witpsol and cocoa butter were selected as individual suppository bases while Tween 80 was used to solubilise the plant extract [18]. Table 1 shows the proportions of materials used in the formulation. For each formulation

containing the extract (SP2 – SP8), a 2/3 portion of the base was gently heated to melt under water bath maintained at a temperature of 37 °C. The remaining un-melted 1/3 portion of the base was added to the melted portion and the mixture stirred gently to homogenize [18]. To this mixture was added previously determined quantity of the extract earlier dispersed in Tween 80 and the resulting mixture stirred again to a viscous consistency. Thereafter, the warm mixture was poured into a 1 g capacity suppository mould which cavities were previously lubricated with liquid paraffin. The set up was allowed to cool to room temperature and then placed in a deep freezer for 48 h after which the formed suppositories were removed and individually wrapped in aluminium foil wrappers.

Table 1: Formulation of *V. amygdalina* extract suppository

Materials (weight %)	SP1	SP2	SP3	SP4	SP5	SP6	SP7	SP8
Extract	-	25	25	25	25	25	35	40
Cocoa butter	-	75	-	-	-	-	-	-
PEG-1000	-	-	75	-	-	-	-	-
PEG-4000	100	-	-	75	-	-	65	60
PEG-6000	-	-	-	-	75	-	-	-
Witpsol	-	-	-	-	-	75	-	-
Total (%)	100	100	100	100	100	100	100	100

Characterization and evaluation of suppositories

Appearance and shape

Samples of the various formulation batches were visually examined for appearance, colour, shapes, homogeneity, spots and cracks.

Determination of uniformity of weight, hardness and pH of suppositories

The British Pharmacopoeia [19] procedure for weight uniformity determination was used for the current study. In brief, 20 non-defective suppositories randomly selected from each batch were weighed together and their average weight determined. Thereafter, each suppository was weighed and the percentage deviation of the individual suppository weights from the average was determined and compared to the official specification for the weight range. Another set of 10 suppositories from each batch were used for

hardness determination using a manual hardness testing equipment (Erweka hardness tester, Pharma Test, Germany).

The pH values of the suppositories were determined as per the procedure reported in Ghazwani *et al.*, [20]. Five suppositories from each batch were digested in warm distilled water (37 ± 0.5 °C). The pH of the filtered digest was taken on a digital pH meter (JENWAY 3410).

Determination of disintegration time of suppositories

The disintegration test study was conducted as per BP official procedures. One suppository was placed in each of the six tubes of a disintegration test equipment (Erweka ZT41, Germany) using simulated vaginal fluid (phosphate buffer pH 4.5) maintained at a 37 ± 0.5 °C as the disintegration test medium. While the machine was running, suppository samples were observed

for complete disintegration as defined by the BP. [21]

Determination of the melting point of suppositories

The USP/NF 36 (USP., 2018) melting point apparatus (ElectroThermal®) was used to determine the melting point of the suppositories. Enough quantity of the suppository matrix was collected into a 10 cm length glass capillary tube by pushing one of the open ends of the tube into the softened suppository body until a 1 cm height of suppository was pushed into the tube [22]. The tube was placed in the temperature regulated melting point apparatus and the temperature adjusted upwards gradually. The temperature at which the suppository portion melted completely was recorded as the melting point of the formulation.

Drug release studies

A slightly modified version of the USP basket apparatus (Electrolab Mumbai) method described in Ghazwani et al [21] was used for the release studies using simulated vaginal fluid. For each formulation, one suppository was placed in an open-ended dialysis membrane bag and the two ends securely tied with a clean thread and placed in the basket of the apparatus. The media temperature was maintained at 37 ± 0.5 °C while basket rotation speed was 20 rpm. Three millilitre (3 ml) of the dissolution medium was withdrawn at 0, 5, 10, 20, 30, 40, 50 and 60 (min) points for spectrophotometric assay after a 25-fold dilution. Sink condition was maintained by replacing the withdrawn sample with equivalent volume of fresh dissolution medium.

Stability studies of the prepared suppositories

The International Conference on Harmonization (ICH) procedure described in Ilomunanya et al. [23] was used to conduct a 90-day (17th June – 17th September 2023) accelerated stability studies for three representative batches, SP2, SP6 and SP8 formulated with cocoa butter, PEG-4000 and Witepsol bases respectively. Five suppositories from each formulation batch were securely wrapped in aluminium foil and deposited in accelerated stability test chamber (Constant Climate Chamber HPP410eco). The test conditions were 40 °C/75 % RH (relative humidity). At days 0, 30 and 90, the

suppositories were removed and evaluated for changes in appearance, melting point, hardness, disintegration, pH and active ingredient content. The values obtained at each test period were compared to the pre-test values earlier determined.

Evaluation of antifungal activities of *V. amygdalina* extract suppositories

The antifungal activities of the *V. amygdalina* leaf extract incorporated into the different suppository formulations were determined against a standard antifungal drug (fluconazole) using slightly modified version of the cup – plate Sabouraud Dextrose Agar diffusion technique reported in Adonu et al. [24]. Clinical isolate of *C. albicans* used for the tests was obtained from the Medical Microbiology Laboratory of the Enugu State University of Science and Technology Teaching Hospital, Parklane, Enugu State, Nigeria. One randomly selected suppository from each batch of the formulations was melted and thoroughly dispersed in warm (40 °C) simulated vaginal fluid to obtain a dilution of 0.2 mg/ml concentration of the extract. A 20 ml sterile Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol was poured into each of nine petri dishes that had been properly labelled to represent each of the nine suppository formulations. The plates were aseptically seeded with 0.1 ml of the clinical isolate (standardized) and left to set at room temperature. Thereafter, a 6 mm diameter cork borer was used to create wells in the inoculated SDA. Using a micropipette, 60 ml of the suppository dispersion was carefully dropped into the agar wells of the first eight Petri dishes. Equal concentration (0.2 mg/ml) and volume of fluconazole dispersion was introduced into the well of the ninth dish to serve as positive control. The set ups were allowed to stand on the bench for 1 h to enable adequate diffusion of the extract/fluconazole and thereafter incubated at 37 °C for 24 h. Subsequently, the inhibition zone diameter of each formulation was observed and measured to the nearest millimetre (mm) using a transparent plastic metre rule.

Data analysis

Data obtained were reported as means and standard deviation (value = mean $\pm$ sd, n = 3). The data were analysed using one-way analysis

of variance (ANOVA) in GraphPad Prism 5.00 software (San Diego, CA, USA) followed by Tukey's post hoc test. Statistically significant differences were accepted at $p < .05$. Tables, figure and charts were also used for the presentation of data and other results.

Results and discussion

Appearance, phytochemical composition and UV-VIS absorbance spectroscopy of *V. amygdalina* extract

The dried and powdered leaves gave total extract yield of 14.02 ± 0.55 %. The extract was dark green in colour and exhibited sticky texture. It did not disperse freely in cold water but did so in Tween 80. Table 2 shows the identified phytochemical constituents and their relative abundances in the extract.

Table 2: Phytochemical composition of the *V. amygdalina* extract

Phytochemical constituent	flavonoids	total phenolic compounds	saponins	tannins	glycosides	alkaloids
Relative abundance	+++	+++	++	+	+	++

The presence of these phytocomponents in *V. amygdalina* extracts had been severally reported and suggested to play principal roles in the observed therapeutic/medicinal effects of the extract [3, 25]. The invitro effectiveness of the extracts as antimalaria, antibacterial, antifungal, hypoglycaemic, anti-inflammatory, antioxidant and cytotoxic agent have been severally reported [26, 27]. Plant phytochemicals are also believed to facilitate metal reduction that take place during biological synthesis of metal nanoparticles [28].

The maximum wavelength ($\lambda_{\max}$) for optimal UV light absorption was determined at $247.61 \pm$

0.56 nm. This value was comparable to 245.3 nm recorded by Muazu et al. [29]. The $\lambda_{\max}$ value was used in the construction of the calibration curve that was in turn used to determine the assay of the extract in the suppository formulations.

Oral acute toxicity study of the *V. amygdalina* extract

Table 3 shows the results obtained in the two phases of the acute toxicity studies after the specified period of observation post treatment.

Table 3: Acute toxicity test results

Parameter	Phase 1 tests			Phase 2 tests		
	1a	2a	3a	1b	2b	3b
Animal group						
No of animals used	1	1	1	1	1	1
Dose of extract administered (mg/kg bw)	10	100	1000	1600	2900	5000
Period of observation (h)	24	24	24	24	24	24
Incidence of morbidity or death	0	0	0	0	0	0*

Key: 0* - zero death but 1 episode of soft stooling

No death or severe signs of morbidity was observed among the experimental and control animal groups. However, the animal that received 5000 (mg/kg b.w) of the extract experienced a single episode of soft stooling. This was not, however confirmed to be due to the ingested extract. These results suggested that the extract was not toxic at the dose levels

administered but was safe within the experimental dose ranges administered. According to the Lorke's test, lethal dose of a medicinal substance is calculated on the bases of the highest dose that caused no death and the lowest dose that caused death of animal (s). The absence of death at all doses was highly suggestive that *V. amygdalina* extract was safe

for the intended use and may be subjected to human clinical trials.

FTIR analysis of extract suppository base and suppository formulation

The compatibility of the proposed formulation components (extract and bases) was investigated by comparing the Fourier transform infrared (FTIR) spectra of the pure extract and that of the prepared suppository. Figure 1 (A - C) show the FTIR spectra of the extract of *V. amygdalina* (A), PEG-4000 only (without extract) (B) and extract + PEG-4000 suppository formulation (C). The tentative assignments of major vibration bands were marked for the spectra. In the spectrum of *V. amygdalina* alone the major peaks were observed at 3362.1 cm^{-1} , 2922.2 cm^{-1} , 2855.1 cm^{-1} , 1718.3 cm^{-1} , 1623.1 cm^{-1} , and 1036.2 cm^{-1} . The band at 3362.1 cm^{-1} was assigned to O–H bond stretching, the peak at 2922.2 cm^{-1} corresponded to asymmetric stretching vibration of C–H of aliphatic CH_2 group, while that of 2855.1 cm^{-1} corresponded to symmetric stretching vibration of C–H of aliphatic CH_2 group. The absorption peak at 1379.1 cm^{-1} was assigned to C–H bend of CH_3 , while the peak at 1036.2 cm^{-1} was assigned to primary alcohol C–O stretch. The band at 1718.3 cm^{-1} corresponded to C=O stretching of saturated aliphatic ketone while the band observed at 1623.1 cm^{-1} represented C=C stretching vibration of an

alkene group. The spectrum for the formulated suppository (extract + PEG-4000) exhibited similar peaks as the spectrum of PEG-4000 alone (figures 1A and 1C). The major bands were observed at 723.1 cm^{-1} , 1155 cm^{-1} , 1461.1 cm^{-1} , 1744.4 cm^{-1} , 2855.1 cm^{-1} and 2922.2 cm^{-1} . The weak band region at 723.1 cm^{-1} might be due to a C–H rock, methyl bending vibration seen only in long chain alkanes, while that at 1461.1 cm^{-1} , was due to C–H bending S or scissoring. The absorption peak at 1155 cm^{-1} corresponded to the C–O stretch of alcohols. The sharp peak at 1744.4 cm^{-1} corresponded to the C=O stretching vibration of the ester carbonyl functional groups of the triglycerides. The peaks at 2922.2 cm^{-1} corresponded to asymmetric stretching vibration of C–H of aliphatic CH_2 group, while that of 2855.1 cm^{-1} corresponded to symmetric stretching vibration of C–H of aliphatic CH_2 group. The presence of similar absorption peaks in both the pure extract and the suppository formulation suggested that the extract existed predominantly as free molecules in the suppository. Absence of any peak at 3362.1 cm^{-1} for O–H bond stretching present in the extract in the formulation may be due to the fact that some molecules in the extract might have solubilised in the base. Identical peaks in both formulation and pure base equally suggested that the bases did not interact chemically with the extract.

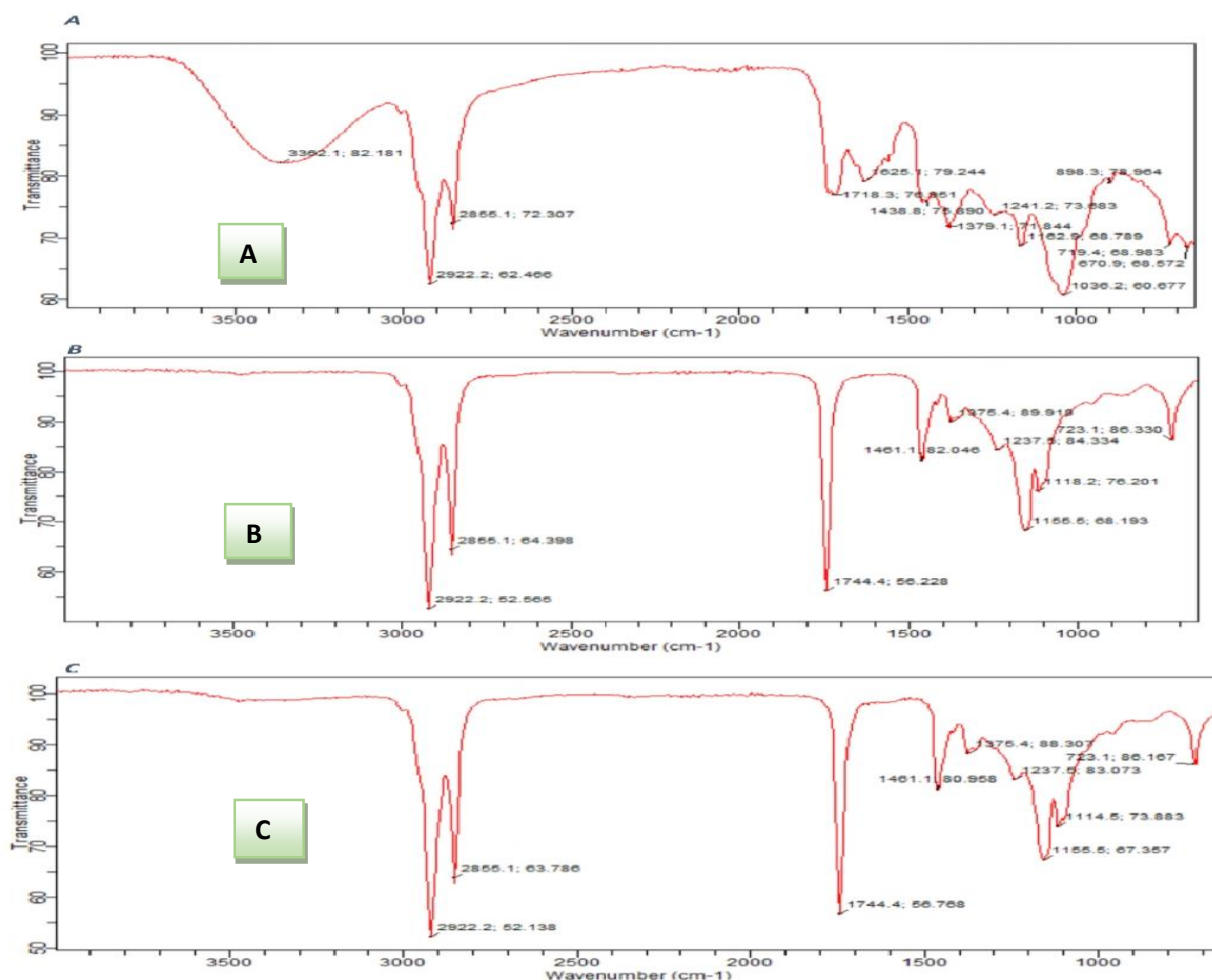


Figure 1: FTIR spectra of A (pure extract of *V. amygdalina*): B (suppository containing PEG-4000 alone) and C (suppository containing *V. amygdalina* extract and PEG-4000)

Physicochemical and organoleptic characteristics of *V. amygdalina* suppositories

Organoleptic characteristics of suppositories.

The suppositories were bullet shaped and dark green in colour except for the control formulation that did not contain the extract. All suppositories had shiny smooth surfaces devoid of sticking, cracks, colour mottling or other physical defects. The suppositories remained in their solid forms after removal from the mould and when wrapped in aluminium foils and stored at room temperature for 30 days. These features

are desirable for suppository formulation which ideally should maintain its solid state at room temperature but melt at the physiologic body temperature, ($37 \pm 0.5^\circ\text{C}$.) to release the active ingredient. They should also maintain their formulation state during handling by patients and upon storage. The torpedo shape of the suppositories facilitates easy vaginal insertion while smooth surfaces avoid risk of vaginal irritation. Figure 2 shows samples of the formulated suppositories.



Figure 2: Formulated vaginal suppositories (SP1: plain base suppositories, SP3: PEG-4000 + 25 %w/w extract, and SP8: PEG 4000 + 40w/w % extract)**Table 4: Physicochemical properties of formulated suppositories**

Batch code	Hardness (kg/cm ²)	pH	MP (°C)	Mean weight (g)	Drug content (%)	DT (min)
SP1	1.8 ± 0.65	6.89	36.01	2.08 ± 0.43	0.00 ± 0.00	13.66 ± 0.48
SP2	2.2 ± 0.33	6.12	37.18	2.06 ± 0.06	94.20 ± 0.32	16.27 ± 0.43
SP3	2.0 ± 0.84	6.24	35.03	2.03 ± 0.11	91.46 ± 0.24	16.02 ± 0.35
SP4	2.3 ± 0.16	6.02	36.07	2.00 ± 0.33	93.88 ± 0.76	19.15 ± 0.51
SP5	3.1 ± 0.05	5.95	37.55	2.09 ± 0.07	92.89 ± 0.07	26.39 ± 0.13
SP6	2.0 ± 0.16	6.45	36.36	2.02 ± 0.38	94.31 ± 0.66	17.71 ± 0.67
SP7	2.3 ± 0.01	6.81	37.31	2.01 ± 0.05	95.04 ± 0.77	21.85 ± 0.04
SP8	2.4 ± 0.12	6.62	37.44	2.00 ± 0.00	97.75 ± 0.43	27.45 ± 0.60

Key: MP = melting point; DT = disintegration time

Physicochemical properties of formulated suppositories

Table 4 shows the mean values of results obtained for various physicochemical parameters from triplicate readings [20].

The hardness values of various extract-containing formulations were in the range of 2.0 ± 0.84 - 3.1 ± 0.05 (kg/cm²). The plain base suppository had hardness value of 1.8 ± 0.65. This observation suggested that the extract exerted some influence on the hardness of the suppositories possibly by enhancing the cohesiveness of the components. Formulation SP5 which contained PEG 6000 (75 %) as main base exhibited the highest hardness of 3.1 ± 0.05 probably due to the additional influence of molecular weight of the PEG. The influence of the molecular weight of PEG on suppository hardness was supported by the values obtained for the three grades of PEG (PEG- 1000, 4000 and 6000) which were in order of PEG-6000 > PEG-4000 > PEG-1000. The optimized formulation, SP8 had a mean hardness value of 2.4 ± 0.12 kg/cm² which was comparable to pharmacopeia specification of 2.0 kg/cm² [30]. This result suggested that the formulation will be capable of withstanding normal handling and transportation stresses and pressures without compromising their structural integrities.

All the formulations exhibited slight acidity with pH range of 5.95 – 6.89. The formulations are, therefore, not likely to cause irritation or unpleasant sensation within the vagina which has natural physiologic pH value of 4.5 - 6.5 (20). Table 4 also shows the melting points of various suppository formulations. Here, melting point was defined as the temperature at which complete liquefaction of the suppositories

occurred. The temperature at which initial melting started was regarded as the softening temperature while the range from the softening point to the complete melting point was defined as the melting range of the suppositories. The melting points of all the formulation were very close to 37 °C. This is the recommended melting point of suppositories since they are required to melt at the normal body temperature to ensure adequate local release of the active ingredients. Suppositories with higher melting temperature may experience delay or inhibition of *in vivo* drug release from the suppository matrix while very low melting temperatures are associated with undesirable liquefaction during storage or handling especially within the tropical climates. It was observed that increasing the concentration of the extract correspondingly increased the melting points. For instance, whereas the MP of formulation SP4 (25 % extract) was 36.07°C, that of formulations SP7 (35 % extract) and SP8 (40 % extract) were 37.31°C and 37.44°C respectively. Plain base formulation (SP1) had MP value of 36.01 °C. However, the formulation containing PEG-6000 had the highest melting point of 37.55 °C.

Weight uniformity of solid dosage forms is vital in ensuring that each unit dose contains equivalent amount of active ingredients. Weight variability may result in over or under dosing of medicines with consequent adverse effects or treatment failures. Weight variation in suppositories may arise from improper (over or under) filling of suppository mould cavities, entrapment of air during mixing or poor blending of suppository materials. The BP specifications require that when the mean weight of 20 randomly selected suppositories is determined,

and the suppositories are thereafter weighed individually, not more than 2 of the individual weights deviate from the average weight by more than 5 % and none deviates by more than 10 %. The mean weights of various formulations are shown in table 4. None of the individual suppository weights deviated by margins outside the pharmacopeial limits.

may constitute barriers to the penetration of the dissolution medium into the matrix and the outward diffusion of the dissolved extract. The formulation exhibited a maximum extract release of 78 % which was attained at 50 min. Slower and prolonged extract release could have continued beyond the test period thereby possibly offering sustained extract delivery with the associated advantages [31]. This will provide additional advantage in the treatment of chronic VVC where frequent dosing and pill burden may constitute impediment to dosing compliance and therapeutic successes. On the contrary, SP3 formulated with PEG-1000 showed extract release of 23 % at the 5th min and 98% release after 30 min of the study. Compared to SP5, it may be concluded that extract release from the suppositories was influenced by the grade (molecular weight) of the PEG. Similarly, the rate and extent of extract release were influenced by the concentration of the extract in the formulation. Formulation SP7 (35% extract)

released 92 % of the drug load at 40 min while SP8 (40 % extract) released 97 % of its content at the same time. It may then be suggested that if in vivo clinical toxicity studies permit, higher strengths of the suppository may be used to increase local drug delivery. Extract release from Witepsol-based suppository (SP6) was comparable to that of SP3(PEG-1000). Both released 98 % of their content in 30 min. These formulations may be appropriate for acute VVC infection where rapid and large loading doses and fast onset of action are desirable to counter spread of infection and exacerbation of symptoms. Cocoa butter base suppositories exhibited the fastest extract release rate. It released up to 97 % of the extract in 30 min and 98 % in 40 min thereby surpassing the release from SP3 (PEG-1000). The possible explanation was that cocoa butter, being an oleaginous fatty base had less tendency to hold the aqueous extracts within the matrix [32]. Generally, all the formulations exhibited adequate extract releases. However, given the need to ensure adequate extract delivery, maintenance of therapeutic concentration at local site of action and desired suppository characteristics, formulation SP8 which contained 40 % of extract and 60 % of PEG-4000 was selected as the optimized formulation and as such recommended for clinical trials.

Table 5: Zones of inhibition of *C. albicans* growth by various suppository formulations

Formulation code	Extract concentration	% Base	Inhibition Zone diameter (mm)
SP1	-	PEG-4000	0.00 ± 0.00
SP2	25	Cocoa butter	13.62 ± 0.34 ^{φ**}
SP3	25	PEG-1000	13.21 ± 0.23 ^{φ*}
SP4	25	PEG-4000	14.05 ± 0.07 ^{φ*}
SP5	25	PEG-6000	13.19 ± 0.65 ^{φ*}
SP6	25	Witepsol	13.86 ± 0.53 ^{φ*}
SP7	35	PEG-4000	17.54 ± 0.43 ^{*ℓ}
SP8	40	PEG-4000	19.86 ± 0.37 ^{*ℓ}
Fluconazole	-	PEG-4000	24.11 ± 0.07

φ**, φ*, *ℓ - no statistically significant difference between inhibition zones of two formulations bearing the same symbol

Evaluation of anti-candida effect of *V. amygdalina* vaginal suppository

The inhibition zone diameter of each suppository formulation is shown on table 5. The data obtained show that all the extract-containing formulations caused *C. albicans* growth inhibition suggesting that the extract has anti-fungal activity. There were no statistically

significant differences ($p < .05$) among the zones of inhibition caused by formulations SP2, SP3, SP4, SP5 and SP6 all of which contained 25 % of extract despite the use of different suppository bases and despite the differences in their extract release rates. The possible reason may be that relatively equivalent quantity of the extract diffused from the various formulations

into the inoculation media in the petri dish. It may, therefore, be suggested that type and grade of base may not have played significant role in the antifungal effectiveness of the extract. Statistically significant differences ($*p < .05$) were observed in the inhibition zones caused by formulations SP2 – SP6 all of which contained 25 % extract with respect to formulations SP7 and SP8 containing 35 and 40 % extracts respectively. Similarly, the difference in the zone of inhibition of SP7 (35 % extract) and SP8 (40 % extract) was statistically significant ($*p < .05$). The implication of this finding is that the anti-candida effect of the *V. amygdalina* extract in the suppositories was dose dependent. While the SP7 formulation caused a zone of inhibition equal to 17.54 ± 0.43 mm, SP8

showed 19.86 ± 0.37 mm zone of inhibition. The differences in the zone of inhibition caused by the positive control (fluconazole) relative to other formulations were also statistically significant at $p < .05$.

Accelerated stability studies of suppository samples

There were no significant differences in the values of the tested quality parameters obtained at day 0 and values obtained at days 30 and 90 test points. Where minor differences were noticed, none fell outside the standard specifications for the parameters at any of the various test points. The comparative values are shown on table 6.

Table 6: Physicochemical parameters of formulations at three stability test points

Testing point	Formulation	Parameter					
		Appearance	Hardness	pH	DT	MP	% CONTENT
Day 0	SP2	No defect	2.21 ± 0.33	6.12	16.27	35.18	94.26
	SP6	No defect	2.03 ± 0.16	6.46	17.71	36.36	94.31
	SP8	No defect	2.45 ± 0.01	6.62	27.45	37.43	97.75
Day 30	SP2	No change	2.22 ± 0.75	6.18	15.07	34.21	93.32
	SP6	No change	2.06 ± 1.13	6.41	17.97	36.78	95.01
	SP8	No change	2.43 ± 0.01	6.59	27.33	38.03	96.42
Day 90	SP2	No change	2.24 ± 0.33	6.09	16.11	33.88	93.82
	SP6	No change	2.09 ± 0.16	6.43	18.21	37.28	93.91
	SP8	No change	2.39 ± 0.01	6.65	28.40	37.17	96.77

CONCLUSION

This work has confirmed the invitro anti *C. albicans* activities of aqueous extract of *V. amygdalina* which had been suggested in previous studies. Delivery of the extract as suppository dosage forms could enhance intra-vaginal delivery and local antifungal activities of the extract. Different grades of PEGs are suitable for the extract suppository formulation. However, large molecular weight PEG caused more gradual releases with potential for modified delivery of the extract. Low molecular weight grades of PEG may be a preferred when rapid delivery and fast onset of action is required. Given the wide availability of *V. amygdalina* plants, the established safety and ethnomedicinal profiles of the leaves and the proven antifungal effectiveness of the extract, the current formulations are recommended for

full clinical trials and eventual commercialization.

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