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Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is a continuation of the former "Eczacılık Bülteni" which was first published in 1953 by Prof. Dr. Kasım Cemal GÜVEN's editorship. At that time, "Eczacılık Bülteni" hosted scientific papers from the School of Medicine-Pharmacy at Istanbul University, Turkey.

In 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and it became a journal for national and international manuscripts, in all fields of pharmaceutical sciences in both English and Turkish. (1984-1995, edited by Prof. Dr. Kasım Cemal GÜVEN, 1995-2001, edited by Prof. Dr. Erden GÜLER, 2002-2011, edited by Prof. Dr. Kasım Cemal GÜVEN)

Since 2006, the journal has been published only in English with the name, "Acta Pharmaceutica Sciencia" which represents internationally accepted high-level scientific standards. The journal has been published quarterly except for an interval from 2002 to 2009 in which its issues were released at intervals of four months. The publication was also temporarily discontinued at the end of 2011 but since 2016, Acta Pharmaceutica Sciencia has continued publication with the reestablished Editorial Board and also with the support of you as precious scientists.

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK

Editor

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An Efficient Approach to the Synthesis of Thymidine Derivatives Containing Various Acyl Groups: Characterization and Antibacterial Activities

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ABSTRACT

In search of new leads toward potent antibacterial agents; therefore, a series of thymidine analogues were synthesized by direct acylation method and furnished the 5´-*O*-acyl thymidine derivatives in good yield. A number of acyl derivatives were prepared in order to obtain a series of newer components for antibacterial screening experiments. The synthesized compounds were characterized by their FTIR, ^{1}HNMR spectral data and elemental analysis. These thymidine derivatives were evaluated for *in vitro* antibacterial screening studies against a number of human pathogenic microorganisms by disc diffusion method. The study revealed that most of the tested chemicals exhibited moderate to good antibacterial activities. It was also observed that the test chemical 2-bromobenzoyl derivative 11 very significantly inhibited the growth of all Gram-positive and Gram-negative bacterial strains used. For comparative studies, antibacterial activity of standard antibiotics, Azithromycin was also carried out against these microorganisms. Hence, these thymidine derivatives can be used to discover antibacterial agents that may serve as leads in the development of new pharmaceuticals research activities.

Keywords: Thymidine, synthesis, derivatives, ^{1}H-NMR, antibacterial

INTRODUCTION

Nucleosides are key compounds involved in major biological processes, such as nucleic acids and proteins synthesis, cell signaling, enzyme regulation, and metabolism. Nucleoside and their derivatives have emerged as molecules with

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potentially useful therapeutic properties that have gained considerable attention from both synthetic and medicinal chemists due to their versatile biological activities in various therapeutic areas. Zidovudine (3′-azido-3′-deoxythymidine) is the first approved drug for the treatment of HIV[1], marketed under the brand name Retrovir. Over the past few years, several derivatives of the nucleosides are known to possess antimicrobial[2], anticancer[3], anti-inflammatory[4] and antiviral activities[5-8].

Thymidine (=deoxythymidine) (Figure 1) is a pyrimidinedeoxy nucleoside. Deoxythymidine is the DNA nucleoside T, which pairs with deoxyadenosine (A) in double-stranded DNA. Since thymine nucleotides are precursors of DNA (but not RNA), the prefix "deoxy" is often left out, i.e., deoxythymidine is often just called thymidine. Since nucleosides and their analogues are of enormous importance. They are an established class of clinically useful medicinal agents possessing antiviral and anticancer activity; at the same time, they are one class of compounds worthy of further investigation as antibacterial agents since some derivatives have shown moderate to good activity against specific bacterial strains[9]. This has led to our interest in the search for new nucleoside i.e., thymidine derivatives that may be screened for broad-spectrum antibacterial activity.

It was revealed that a large number of biologically active compounds possess aromatic, heteroaromatic and acyl substituents[10]. Also, a wide variety of compounds having nitrogen, sulphur and halogen containing substituents possess effective biological activity[10-12]. It is also known that if an active nucleus is linked to another active nucleus, the resulting molecule may possess greater potential for biological activity[13]. From our previous works we also observed that in many cases the combination of two or more acyl substituents in a single molecular framework enhances the biological activity manyfold than their parent nuclei[14-18]. Encouraged by our own findings and also literature reports, we synthesised a series of thymidine derivatives deliberately incorporating a wide variety of anticipated biologically active components to the deoxyribose moiety. Antibacterial activities of these compounds were carried out using a variety of bacterial strains and the results are reported here as first time.

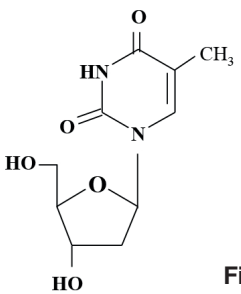


Figure 1. Thymidine (Compound 1)

METHODOLOGY

Chemistry

FTIR spectra were recorded by KBr disc at the Chemistry Department, University of Chittagong, Bangladesh, with an IR Affinity Fourier Transform Infrared Spectrophotometer (SHIMADZU, Japan). All reagents used were commercially available (Sigma-Aldrich) and were used as received, unless otherwise specified. Melting points were determined on an electro-thermal melting point apparatus (England) and are uncorrected. Evaporations were carried out under reduced pressure using VV-1 type vacuum rotary evaporator (Germany) with a bath temperature below 40°C. ^{1}H-NMR spectra (400 MHz) were recorded for solutions in deuteriochloroform ($CDCl_3$) (internal Me_4Si) with a Bruker DPX-40C spectrometer. Thin layer chromatography (t.l.c) was performed on Kieselgel GF_{254} and spots were detected by spraying the plates with 1% H2SO4 and heating at 150-2000C until coloration took place. Column chromatography was performed with silica gel G_{60}. Solvent system employed for TLC analyses was methanol-chloroform in different proportions.

Synthesis

Although studies for the synthesis of the nucleosides began[19] in 1948, the preparations of the nucleosides and their analogues are still a particularly challenging and attractive target for the synthetic community because of their promising pharmacological profiles.

5´-*O*-(Acetyl)thymidine (Compound 2)

A suspension of thymidine (1) (200 mg, 0.82 mmol) in dry pyridine (3 ml) was cooled to -50C where upon acetic anhydride (0.0848 ml, 1.1 molar eq.) was added to it. The mixture was stirred at this temperature for 6 hours and then stirred overnight at room temperature. The reaction progress was monitored by TLC (methanol-chloroform 1:5), which indicated full conversion of the starting material into a single product (R_f =0.52).

A few pieces of ice were then added to the reaction flask with constant shaking to destroy the excess reagent and the contents were extracted with chloroform (3x10 mL). The combined chloroform layer was washed successively with dilute hydrochloric acid (10%), saturated aqueous sodium hydrogen carbonate ($NaHCO_3$) solution and distilled water. The chloroform layer was dried with anhydrous magnesium sulphate ($MgSO_4$), filtered and the filtrate was concentrated under reduced pressure to leave a syrup. The syrup was passed through a silica gel column chromatography and eluted with methanol-

chloroform (1:5) provided the acetyl chloride derivative (2) (141 mg) as semi solid. The compound was sufficiently pure for use in the next stage without further purification and identification.

FTIR (KBr) ν_{max} (cm^{-1}): 1684 (-CO), 3430 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9. 01 (1H, s, -NH), 7.31 (1H, d, J=1.3 Hz, H-6), 6.29 (1H, m, H-1´), 4.22-4.20 (1H, m, H-3´), 4.18 (1H, dd, J=12.0 and 4.7 Hz, H-5´a), 4.11 (1H, dd, J=12.1 and 4.5 Hz, H-5´b), 4.05 (1H, ddd, J=3.6, 4.6 and 4.2 Hz, H-4´), 3.37 (1H, br s, 3´-OH), 2.35 (1H, ddd, J=13.7, 6.6 and 4.4 Hz, H-2´a), 2.25 (1H, ddd, J=13.6, 6.6 and 6.8 Hz, H-2´b), 2.11 (3H, s, CH_3CO-), 1.72 (3H, d, J = 1.3 Hz, 5-CH_3). Anal. calcd for $C_{11}H_{17}O_5N_2CO$ (289.26): C, 45.63; H, 5.87. Found: C, 45.65; H, 5.88.

General synthesis of thymidine derivatives

A solution of thymidine (1) (200 mg, 0.82 mmol) in anhydrous pyridine (3 ml) was cooled to 00C when pentanoyl chloride (0.1077 mL, 1.1 molar eq.), heptanoyl chloride (0.1336 mL, 1.1 molar eq.), octanoyl chloride (0.1534 mL, 1.1 molar eq.), decanoyl chloride (0.1846 mL, 1.1 molar eq.), myristoyl chloride (0.8256 mL, 1.1 molar eq.), pivaloyl chloride (0.1106 mL, 1.1 molar eq.), 2-chlorobenzoyl chloride (0.1092 mL, 1.1 molar eq.), 2-bromobenzoyl chloride (0.1163 mL, 1.1 molar eq.), 4-bromobenzoyl chloride (0.195 mL, 1.1 molar eq.) and cinnamoyl chloride (0.1482 mL, 1.1 molar eq.) were separately added to it, respectively. The mixture was stirred at 0°C for 6~7 hours and then overnight at room temperature. T.L.C. examination (methanol-chloroform, 1:5) showed complete conversion of reactant into a single product. A few pieces of ice were added to the reaction flask in order to destroy the excess reagent and the reaction mixture was processed as usual. Percolation of the resulting syrup by passage through a silica gel column with methanol-chloroform, (1:5), as eluant afforded the pentanoyl derivative (3) (148 mg) as a semi-solid mass which could not be crystallized. Similarly isolate the compound 4 (137 mg), compound 5 (162 mg), compound 6 (152 mg), compound 7 (156 mg), compound 8 (152 mg), compound 9 (159 mg), compound 10 (125 mg), compound 11 (138 mg), compound 12 (145 mg) and compound 13 (165 mg), successfully.

5´-*O*-(Pentanoyl)thymidine (Compound 3)

FTIR (KBr) ν_{max} (cm^{-1}): 1694 (-CO), 3432 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9. 01 (1H, s, -NH), 7.27 (1H, d, J=1.3 Hz, H-6), 6.24 (1H, t, J=6.5 Hz, H-1´), 4.40-4.32 (1H, m, H-3´), 4.40 (1H, dd, J=12.0 and 4.5 Hz, H-5´a), 4.25 (1H, dd, J=12.0 and 3.5 Hz, H-5´b), 4.15 (1H, ddd, J=3.5, 4.5 and 3.9 Hz, H-4´), 3.47 (1H, br, 3´-OH), 2.38 (1H, ddd, J=13.5, 6.5 and 4.0 Hz, H-2´a), 2.36 {2H, m, $CH_3(CH_2)_2CH_2CO$-}, 2.25 (1H, ddd, J=13.5, 6.5 and 6.7 Hz, H-2´b),

1.92 (3H, d, J=1.3 Hz, 5-CH_3), 1.64 (2H, m, $CH_3CH_2CH_2CH_2CO$-), 1.26 {2H, m, $CH_3CH_2(CH_2)_2CO$-}, 0.88 {3H, m, $CH_3(CH_2)_3CO$-}. Anal. calcd for $C_{15}H_{23}O_6N_2$ (326.346): C, 55.15; H, 7.11. Found: C, 55.18; H, 7.12.

5´-*O*-(Heptanoyl)thymidine (Compound 4)

FTIR (KBr) ν_{max} (cm^{-1}): 1706 (-CO), 3465 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9. 00 (1H, s, -NH), 7.17 (1H, d, J=1.3 Hz, H-6), 6.82 (1H, t, J=6.5 Hz, H-1´), 4.30 (1H, m, H-3´), 4.27 (1H, dd, J=12.0 and 4.5 Hz, H-5´a), 4.20 (1H, dd, J=12.0 and 3.5 Hz, H-5´b), 4.01 (1H, ddd, J=3.5, 4.5 and 3.9 Hz, H-4´), 3.35 (1H, s, 3´-OH), 2.36 (1H, ddd, J=13.5, 6.5 and 4.0 Hz, H-2´a), 2.33 {2H, m, $CH_3(CH_2)_4CH_2CO$-}, 2.25 (1H, ddd, J=13.5, 6.5 and 6.7 Hz, H-2´b), 1.93 (3H, d, J=1.3 Hz, 5-CH_3), 1.64 {2H, m, $CH_3(CH_2)_3CH_2CH_2CO$-}, 1.28 {6H, m, $CH_3(CH_2)_3CH_2CH_2CO$-}, 0.89 {3H, m, $CH_3(CH_2)_5CO$-}. Anal. calcd for $C_{16}H_{23}O_5N_2CO$ (354.399): C, 54.17; H, 6.48. Found: C, 54.20; H, 6.51.

5´-*O*-(Octanoyl)thymidine (Compound 5)

FTIR (KBr) ν_{max} (c^{m-1}): 1731 (-CO), 3400 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 8. 98 (1H, s, -NH), 7.12 (1H, d, J=1.8 Hz, H-6), 6.80 (1H, t, J=6.6 Hz, H-1´), 4.43 (1H, m, H-3´), 4.33 (1H, dd, J=12.0 and 4.5 Hz, H-5´a), 4.28 (1H, dd, J=12.2 and 3.6 Hz, H-5´b), 4.10 (1H, m, H-4´), 3.41 (1H, br s, 3´-OH), 2.40 (1H, ddd, J=13.6, 6.6 and 4.6 Hz, H-2´a), 2.24 (1H, ddd, J=12.5, 6.1 and 6.2 Hz, H-2´b), 1.92 (3H, d, J=1.3 Hz, 5-CH_3), 2.36 {2H, m, $CH_3(CH_2)_5CH_2CO$-}, 1.61 {2H, m, $CH_3(CH_2)_4CH_2CH_2CO$-}, 1.24 {8H, m, $CH_3(CH_2)_4(CH_2)_2CO$-}, 0.86 {3H, m, $CH_3(CH_2)_6CO$-}. Anal. calcd for $C_{17}H_{29}O_5N_2CO$ (368.429): C, 55.37; H, 7.87. Found: C, 55.39; H, 7.90.

5´-*O*-(Decanoyl)thymidine (Compound 6)

FTIR (KBr) ν_{max} (cm^{-1}): 1701 (-CO), 3399 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 8. 98 (1H, s, -NH), 7.17 (1H, d, J=2.3 Hz, H-6), 6.22 (1H, t, J=6.5 Hz, H-1´), 4.28 (1H, m, H-3´), 4.41 (1H, dd, J=11.8 and 4.6 Hz, H-5´a), 4.20 (1H, dd, J=12.1 and 4.5 Hz, H-5´b), 4.01 (1H, ddd, J=4.5, 5.5 and 4.9 Hz, H-4´), 3.41 (1H, s, 3´-OH), 2.36 (1H, ddd, J=13.0, 6.8 and 4.5 Hz, H-2´a), 2.32 {2H, m, $CH_3(CH_2)_7CH_2CO$-}, 2.20 (1H, ddd, J=13.6, 6.2 and 6.8 Hz, H-2´b), 1.90 (3H, d, J=1.3 Hz, 5-CH_3), 1.64 {2H, m, $CH_3(CH_2)_6CH_2CH_2CO$-}, 1.24 {12H, m, $CH_3(CH_2)_6CH_2CH_2CO$-}, 0.87 {3H, m, $CH_3(CH_2)_8CO$-}. Anal. calcd for $C_{19}H_{33}O_5N_2CO$ (396.479): C, 57.50; H, 8.32. Found: C, 57.52; H, 8.34.

5´-*O*-(Myristoyl)thymidine (Compound 7)

FTIR (KBr) ν_{max} (cm^{-1}): 1702 (-CO), 3398 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δH 8. 61 (1H, s, -NH), 7.27 (1H, d, J=1.3 Hz, H-6), 6.24 (1H, t, J=6.5 Hz, H-1´),

4.40-4.32 (1H, m, H-3´), 4.40 (1H, dd, J=12.0 and 4.5 Hz, H-5´a), 4.25 (1H, dd, J=12.0 and 3.5 Hz, H-5´b), 4.15 (1H, ddd, J = 3.5, 4.5 and 3.9 Hz, H-4´), 3.47 (1H, br, 3´-OH), 2.38 (1H, ddd, J = 13.5, 6.5 and 4.0 Hz, H-2´a), 2.34 {2H, m, $CH_3(CH_2)_{11}CH_2CO$-}, 2.25 (1H, ddd, J=13.5, 6.5 and 6.7 Hz, H-2´b), 1.92 (3H, d, J=1.3 Hz, 5-CH_3), 1.74-1.48 {2H, m, $CH_3(CH_2)_{10}CH_2CH_2CO$-}, 1.42-1.14 {20H, br m, $CH_3(CH_2)_{10}CH_2CH_2CO$-}, 0.86 {3H, t, J = 6.8 Hz, $CH_3(CH_2)_{12}CO$-}. Anal. calcd for $C_{23}H_{41}O_5N_2CO$ (452.589): C, 60.98; H, 9.05. Found: C, 60.99; H, 9.08.

5´-*O*-(Pivaloyl)thymidine (Compound 8)

FTIR (KBr) ν_{max} (cm^{-1}): 1708 (-CO), 3408 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9. 00 (1H, s, -NH), 7.31 (1H, d, J=2.3 Hz, H-6), 6.25 (1H, t, J=6.6 Hz, H-1´), 4.20 (1H, m, H-3´), 4.41 (1H, dd, J=12.0 and 4.5 Hz, H-5´a), 4.25 (1H, m, H-5´b), 3.94 (1H, m, H-4´), 3.46 (1H, s, 3´-OH), 2.38 (1H, ddd, J=13.5, 6.5 and 4.0 Hz, H-2´a), 2.25 (1H, ddd, J=13.5, 6.5 and 6.7 Hz, H-2´b), 1.93 (3H, d, J=1.3 Hz, 5-CH_3), 1.22 {9H, s, $(CH_3)_3CCO$-}. Anal. calcd for $C_{14}H_{23}O_5N_2CO$ (326.349): C, 51.47; H, 7.04. Found: C, 51.49; H, 7.07.

5´-*O*-(Benzenesulphonyl)thymidine (Compound 9)

FTIR (KBr) ν_{max} (cm^{-1}): 1735 (-CO), 3378 (-OH), 1365 (-SO_2). ^{1}H-NMR (400 MHz, CDCl3): δ_H 8. 92 (1H, s, -NH), 7.88 (2H, m, Ar-H), 7.75 (1H, m, Ar-H), 7.60 (2H, m, Ar-H), 7.14 (1H, d, J=1.2 Hz, H-6), 6.82 (1H, t, J=6.4 Hz, H-1´), 4.41 (1H, m, H-3´), 4.22 (1H, dd, J=12.1 and 4.4 Hz, H-5´a), 4.19 (1H, dd, J=12.0 and 4.5 Hz, H-5´b), 3.96 (1H, ddd, J=3.4, 4.4 and 3.7 Hz, H-4´), 3.36 (1H, s, 3´-OH), 2.83 (1H, ddd, J=13.5, 6.5 and 4.0 Hz, H-2´a), 2.68 (1H, ddd, J=13.5, 6.5 and 6.7 Hz, H-2´b), 1.96 (3H, d, J=1.3 Hz, 5-CH_3). Anal. calcd for $C_{16}H_{19}O_5N_2SO_2$ (382.389): C, 50.21; H, 4.96. Found: C, 50.24; H, 4.99.

5´-*O*-(2-Chlorobenzoyl)thymidine (Compound 10)

FTIR (KBr) ν_{max} (cm^{-1}): 1701 (-CO), 3402 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9. 01 (1H, s, -NH), 7.78 (1H, m, Ar-H), 7.61 (2H, m, Ar-H), 7.28 (1H, m, Ar-H), 7.15 (1H, d, J=1.7 Hz, H-6), 6.80 (1H, t, J=6.6 Hz, H-1´), 4.21 (1H, m, H-3´), 4.19 (1H, dd, J=12.1 and 4.7 Hz, H-5´a), 4.15 (1H, dd, J=11.9 and 3.8 Hz, H-5´b), 4.00 (1H, ddd, J=3.7, 4.8 and 4.2 Hz, H-4´), 3.35 (1H, s, 3´-OH), 2.86 (1H, m, H-2´a), 2.65 (1H, m, H-2´b), 1.72 (3H, d, J=1.6 Hz, 5-CH3). Anal. calcd for $ClC_{16}H_{17}N_2O_5CO$ (379.776): C, 50.60; H, 4.51. Found: C, 50.65; H, 4.54.

5´-*O*-(2-Bromobenzoyl)thymidine (Compound 11)

FTIR (KBr) ν_{max} (cm^{-1}): 1702 (-CO), 3399 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 8. 71 (1H, s, -NH), 7.81 (1H, d, J=7.8 Hz, Ar-H), 7.27 (1H, d, J=1.3 Hz, H-6), 7.67 (2H, m, Ar-H), 7.42 (1H, m, Ar-H), 6.28 (1H, t, J=6.7 Hz, H-1´), 4.43-4.29 (1H,

m, H-3´), 4.54 (1H, dd, J=12.1 and 4.6 Hz, H-5´a), 4.25 (1H, dd, J=12.1 and 3.7 Hz, H-5´b), 4.19 (1H, ddd, J=3.5, 4.6 and 4.1 Hz, H-4´), 3.47 (1H, br s, 3´-OH), 2.42 (1H, ddd, J=13.6, 6.5 and 4.2 Hz, H-2´a), 2.25 (1H, ddd, J=13.5, 6.7 and 6.8 Hz, H-2´b), 1.92 (3H, d, J=1.6 Hz, 5-CH_3). Anal. calcd for $BrC_{16}H_{18}O_5N_2CO$ (425.229): C, 45.15; H, 4.23. Found: C, 45.18; H, 4.26.

5´-*O*-4-(Bromobenzoyl)thymidine (Compound 12)

FTIR (KBr) ν_{max} (cm^{-1}): 1698 (-CO), 3396 (-OH). ^{1}H-NMR (400 MHz, $CDCl_3$): δH 9. 00 (1H, s, -NH), 7.91 (2H, m, Ar-H), 7.61 (2H, m, Ar-H), 7.21 (1H, d, J=1.8 Hz, H-6), 6.82 (1H, t, J=6.6 Hz, H-1´), 4.52 (1H, m, H-3´), 4.39 (1H, dd, J=11.8 and 4.3 Hz, H-5´a), 4.18 (1H, dd, J=12.0 and 3.6 Hz, H-5´b), 4.10 (1H, ddd, J=3.6, 4.7 and 4.2 Hz, H-4´), 3.36 (1H, s, 3´-OH), 2.74 (1H, ddd, J=13.1, 6.4 and 4.1 Hz, H-2´a), 2.12 (1H, ddd, J=13.2, 6.3 and 6.6 Hz, H-2´b), 1.78 (3H, d, J=1.6 Hz, 5-CH_3). Anal. calcd for $BrC_{16}H_{18}O_5N_2CO$ (425.231): C, 45.15; H, 4.23. Found: C, 45.19; H, 4.25.

5´-*O*-(Cinnamoyl)thymidine (Compound 13)

FTIR (KBr) ν_{max} (cm^{-1}): 1735 (-CO), 3400 (-OH), 1628 (-CH=CH-). ^{1}H-NMR (400 MHz, $CDCl_3$): δH 9. 31 (1H, s, -NH), 7.75 (1H, d, J=12.0 Hz, PhCH=CHCO-), 7.51 (2H, m, Ar-H), 7.40 (1H, d, J=1.8 Hz, H-6), 7.11 (3H, m, Ar-H), 6.82 (1H, d, J=12.1 Hz, PhCH=CHCO-), 6.44 (1H, t, J=6.4 Hz, H-1´), 4.45 (1H, m, H-3´), 4.23 (1H, dd, J=12.1 and 4.5 Hz, H-5´a), 4.18 (1H, dd, J=12.0 and 3.6 Hz, H-5´b), 3.96 (1H, ddd, J=3.8, 4.8 and 3.8 Hz, H-4´), 3.35 (1H, br s, 3´-OH), 2.44 (1H, ddd, J=13.4, 6.4 and 4.4 Hz, H-2´a), 2.15 (1H, ddd, J=13.5, 6.6 and 6.8 Hz, H-2´b), 1.90 (3H, d, J=1.6 Hz, 5-CH_3). Anal. calcd for $C_{18}H_{21}O_5N_2CO$ (372.369): C, 58.0; H, 5.64. Found: C, 58.03; H, 5.66.

Tested Bacterial pathogens

Test tube cultures of bacterial pathogens were obtained from the Department of Biochemistry and Molecular Biology, University of Chittagong. The synthesized test compounds (Scheme 1 & 2) were subjected to antibacterial screening against two Gram-positive (*Bacillus subtilis* BTCC 17 & *Bacillus cereu*s BTCC 19) and two Gram-negative (*Escherichia coli* ATCC 25922 & *Salmonella paratyphi* AE 14612) bacterial strains (Table 1).

Table 1. List of used bacteria

Types of bacteria	Name of tested bacteria	Strain no.
Gram +Ve	*Bacillus subtilis* *Bacillus cereus*	BTCC 17 BTCC 19
Gram -Ve	*Escherichia coli* *Salmonella paratyphi*	ATCC 25922 AE 146313

Antibacterial assay

The *in vitro* antibacterial spectrum of the newly synthesized thymidine derivatives (2-13) was done by disc diffusion method[20] with little modification[21]. Sterilized paper discs of 4 mm in diameter and Petri dishes of 150 mm in diameter were used throughout the experiment. The autoclaved Mueller-Hinton agar medium, cooled to 45˚C, was poured into sterilized Petri dishes to a depth of 3 to 4 mm and after solidification of the agar medium; the plates were transferred to an incubator at 37˚C for 15 to 20 minutes to dry off the moisture that developed on the agar surface. The plates were inoculated with the standard bacterial suspensions (as McFarland 0.5 standard) followed by spread plate method and allowed to dry for three to five minutes. Dried and sterilized filter paper discs were treated separately with 50 µg dry weight/disc from 2% solution (in $CHCl_3$) of each test chemical using a micropipette, dried in air under aseptic condition and were placed at equidistance in a circle on the seeded plate. A control plate was also maintained in each case without any test chemical. These plates were kept for 4-6 hours at low temperature (4-6˚C) and the test chemicals diffused from disc to the surrounding medium by this time. The plates were then incubated at 35±2˚C for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter. Each experiment was repeated thrice. All the results were compared with the standard antibacterial antibiotic ampicillin (20 µg/disc, BEXIMCO Pharm Bangladesh Ltd).

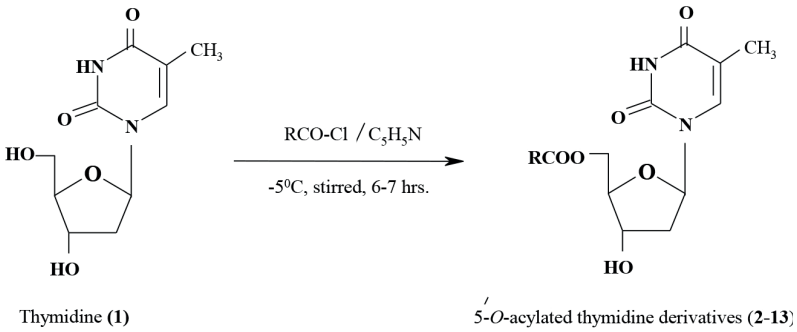
RESULTS AND DISCUSSION

Synthesis and spectroscopic characterization

In the present study, selective acylation of thymidine (1) was performed using the direct method. The structure of the acylated products were ascertained by analyzing their FTIR and ^{1}H-NMR spectra[22-24]. The reaction pathways have been summarized in the Scheme 1- 2 and Table 2.

Our initial effort was reacted thymidine (1) with unimolecular amount of acetic anhydride as acylating agent in dry pyridine at freezing temperature, followed by removal of solvent and silica gel column chromatographic purification, furnished the 5′-*O*-acetyl derevative (2). The FTIR spectrum of compound 2 showed the following absorption bands: 1684 cm^{-1} (due to -CO), 3430 cm^{-1} and (due to –OH) and stretchings. In its ^{1}H-NMR spectrum one three-proton singlet at δ 2.11 was due to the methyl protons of one acetyloxy group. The downfield shifts of H-5′ to δ 4.18 (as dd, J = 12.0 and 4.7 Hz, H-5´a) and 4.11 (as, dd, J = 12.1 and 4.5 Hz, H-5´b) as compared to the usual values indicated the attachment of the acetyl

group at position 5′. Complete analysis of the rest of the FTIR and 1H-NMR spectra was in supported the structure ascertained as 5′-*O*-(acetyl)thymidine (2). This finding was in conformity with the mechanism proposed by Parang et al.[25] based on similar thymidine derivatives.



Thymidine **(1)** 5′-*O*-acylated thymidine derivatives (**2**-**13**)

Scheme 1. Synthetic pathway followed for the preparation of thymidine derivatives

Compound no.	R	Compound no.	R
2	CH_3CO-	**8**	$(CH_3)_3CCO$-
3	$CH_3(CH_2)_3CO$-	**9**	$C_6H_5SO_2$-
4	$CH_3(CH_2)_5CO$-	**10**	2-Cl.C_6H_4CO-
5	$CH_3(CH_2)_6CO$-	**11**	2-Br.C_6H_4CO-
6	$CH_3(CH_2)_8CO$-	**12**	4-Br.C_6H_4CO-
7	$CH_3(CH_2)_{12}CO$-	**13**	$C_6H_5CH{=}CHCO$-

Scheme 2. Structure of thymidine derivatives (Compounds 2-13)

Further reaction was achieved by its conversion to the pentanoate derivative **3.** In its ^{1}H-NMR spectrum, the resonance peaks three two-proton multiplets at δ 2.36 {$CH_3(CH_2)_2CH_2CO$-}, δ 1.64 {$CH_3CH_2CH_2CH_2CO$-} and δ 1.24 {$CH_3CH_2(CH_2)_2CO$-} and one three-proton multiplet at δ 0.88 {$CH_3(CH_2)_3CO$-} showed the presence of one pentanoyl group in the compound. The deshielding of C-5/ protons to δ 4.40 (as dd, J = 12.0 and 4.5 Hz, H-5′a) and 4.25 (as dd, J = 12.0 and 3.5 Hz, H-5′b) indicated the introduction of the one pentanoyl group at position 5′. Heptanoylation of the thymidine **(1)** with heptanoyl chloride in dry C_5H_5N using the conventional work-up and purification procedure provided the heptanoate derivative **(4).** The FTIR, ^{1}H-NMR and elemental analysis enabled us to assign the structure of the heptanoyl derivative as 5′-*O*-(heptanoyl)thymidine **(4).** The same thymidine **1** was then converted to the octanoyl and decanoyl derivatives **(5 & 6)**. The structure of the octanoyl and decanoyl derivatives **(5**

& 6) were confidently established by completely analyzing their FTIR, ^{1}H-NMR and elemental data as 5′-*O*-(octanoyl)thymidine **(5)** and 5′-*O*-(decanoyl) thymidine **(6).**

Table 2. Physicochemical properties of the synthesized of thymidine derivatives (2-13).

Compound no	RT (h)	Rf	Yield (%)	Physical State
2	6.0	0.52	70.5	semi solid
3	6.5	0.50	74.0	semi solid
4	6.0	0.52	68.5	liquid syrup
5	6.0	0.51	81.0	thick syrup
6	6.0	0.50	76.0	needles, m.p. 120-1250°C
7	6.5	0.52	78.0	semi solid
8	7.0	0.53	76.0	liquid
9	6.0	0.51	79.5	pasty mass
10	6.5	0.52	62.5	pasty mass
11	6.5	0.51	69.0	pasty mass
12	6.0	0.49	72.5	semi solid mass
13	6.0	0.51	82.5	semi solid

Thymidine **(1)** on treatment with myristoyl chloride, afforded compound **7** and its FTIR spectrum the absorption bands at 1702 cm^{-1} for C=O stretching and 3398 cm^{-1} for -OH stretching. The ^{1}H-NMR spectrum displayed a two two-proton multiplet at δ 2.34 {$CH_3(CH_2)_{11}CH_2CO$-}, and δ 1.74-1.48 {$CH_3(CH_2)_{10}CH_2CH_2CO$-}, a twenty-proton multiplet at δ 1.42-1.14 {$CH_3(CH2)_{10}CH_2CH_2CO$-}, and a three-proton triplet at δ 0.86 {$CH_3(CH_2)_{12}CO$-} suggested the attachment of one myristoyl group in the compound. The rest of the ^{1}H-NMR spectrum was in conformity with the structure accorded to it. The formation of compound **7** may be explained by assuming that myristoyl chloride attaches with the most reactive and less sterically hindered primary hydroxyl group of the ribose moiety at 5/ position, thereby forming the 5′-*O*-(myristoyl) thymidine **(7)** as the sole product.

In the ^{1}H-NMR spectrum of the compound **8** showed a nine-proton singlet at δ 1.22 {$(CH_3)_3CCO$-} was due to the methyl protons of pivaloyl group which indicated the introduction of a pivaloyl group. The downfield shift of H-5/ proton to δ 4.41 (1H, dd, J = 12.0 and 4.5 Hz, H-5′a), and δ 4.25 (as m) from their precursor value and these δ values showed the attachment of the pivaloyl group at position 5/. The rest of the FTIR and 1H-NMR spectra was compatible with the structure assigned as 5/-*O*-(pivaloyl)thymidine **(8).**

Thus, treatment of compound **1** with benzenesulfonyl chloride in dry pyridine followed by usual work-up procedure. The FTIR spectrum of this compound showed the following absorption bands: 1735 cm^{-1} (C=O stretching), 3378 cm^{-1} (-OH stretching) and 1365 cm^{-1} (-SO_2 stretching). In its ^{1}H-NMR spectrum the peaks at δ 7.88 (2H, m), δ 7.75 (1H, m) and δ 7.60 (2H, m) corresponded the protons of one phenyl group. The downfield shift of H-5′ to δ 4.22 (as dd, J = 12.1 and 4.4 Hz, H-5´a) and δ 4.19 (as dd, J = 12.0 and 4.5 Hz, H-5´b) from their usual values ascertained the attachment of benzenesulfonyl group at position 5′. By complete analysis of the FTIR, 1H-NMR and elemental data, the structure of the compound was ascertained as 5′-*O*-(benzenesulphonyl)thymidine **(9).**

Encouraged by the results obtained, we performed 2-chlorobenzoylation of compound **1** using similar procedures and isolated compound **10** in (125 mg, 62.5%) as a pasty mass. The FTIR and ^{1}H-NMR spectrum was in accord with the structure of this compound assigned as 5´-*O*-(2-chlorobenzenzoyl) thymidine **(10).** The formation of a monosubstitution product **11** was clearly revealed by its ^{1}H-NMR spectrum which showed one one-proton doublet at δ 7.81 (as d, J = 7.8 Hz), one two-proton multiplet at δ 7.67 (2H, m) and one one-proton multiplet at δ 7.42 (1H, m) corresponding to the aromatic ring protons of one 2-bromobenzoyl group in the molecule. Complete analysis of the FTIR, ^{1}H-NMR of this compound was in agreement with the structure accorded as 5′-*O*-(2-bromobenzoyl)thymidine **(11).** As same as reaction of compound **1** with 4-bromobenzoyl chloride in dry C_6H_5N, as usual procedure and purification gave the 4-bromobenzoyl derivative **(12).**

Finally, we have carried out cinnamoylation of **1** with an excess of cinnamoyl chloride in pyridine as same work-up and purification techniques, we isolated compound **(13)** in (165 mg, 82.5%) as a semi solid. FTIR spectrum showed absorption bands at 1735 cm^{-1} (for -CO stretching), 3400 cm^{-1} (for -OH-stretching) and 1628 cm^{-1} (for -CH=CH- stretching). In the ^{1}H-NMR spectrum one one-proton doublet at δ 7.75 (as d, J = 12.0 Hz, PhCH=CHCO-) and also one one-proton doublet at δ 6.82 (as d, J = 12.1 Hz, PhCH=C*H*CO-) due to the presence of one cinnamoyl group in the molecule. In addition a two-proton multiplet at δ 7.51 (as m, Ar-H) and a three-proton multiplet at δ 7.11 (as, m, Ar-H) due to the one aromatic ring protons. The downfield shift of C-5 to δ 4.23 (as dd) and δ 4.18 (as dd) from their usual values in the precursor compound 1 and the resonances of other protons in their anticipated positions, showed the presence of the cinnamoyl group at position 5´. The rest of the FTIR and ^{1}H-NMR was in accord with the structure of this compound assigned as *5´-O*-(cinnamoyl)thymidine **(13)**. Thus, selective acylation of thymidine **(1)**

with a number of acylating agents by using the direct method was carried out successfully. The study was found to be very promising since all the reactions; a single monosubstitution product was isolated in reasonably high yield.

Antibacterial activity

The results of antibacterial screening of the test chemicals and the standard antibiotic, Azithromycin are furnished in Table 3 and Figure 2-3. The results revealed that most of the derivatives were prone to antibacterial action against most of the Gram-positive and Gram-negative bacteria. The results exhibited that the test compounds **9** and **13** were highly active towards the growth of all the Gram-positive bacteria. Compound **10** and **11** were completely insensitive towards any of the Gram-positive bacteria.

Table 3. Zone of inhibition observed against Gram+Ve and Gram-Ve test organisms by the thymidine derivatives.

Compound no.	Zone of inhibition (mm) at 200 µg dw/disc			
	Gram +Ve bacteria		Gram -Ve bacteria	
	B. subtilis	*B. cereus*	*E. coli*	*S. paratyphi*
2	NF	5	NF	NF
3	*14	8	*15	7
4	8	7	8	*11
5	10	9	10	8
6	*12	6	*13	9
7	6	*12	6	*12
8	5	6	7	7
9	*13	*14	*12	*11
10	NF	NF	5	NF
11	NF	NF	NF	NF
12	7	8	8	6
13	*12	10	9	9
** Azithromycin	*22	*18	*22	*20

N.B: '*' = marked inhibition, '**' = standard antibiotic, 'NF' = not found, 'dw' = dry weight.

The results showed that except the test chemicals **2, 11** all other test chemicals were found to be effective towards the different Gram-negative bacteria in different degrees. The test chemical **3, 6** and **9** very significantly inhibited the growth of all Gram-negative bacterial strains used.

The inhibition of *E. coli* by **3** (15 mm), **6** (13 mm), **9** (12 mm), of *S. paratyphi* by **4** (11 mm), **7** (12 mm), **9** (11 mm), were remarkable. The inhibitions of growth of

bacteria were very remarkable in many cases which were in conformity with our previous work[26-29]. However, compound 11 was found insensitive towards all the Gram-positive and Gram-negative bacteria.

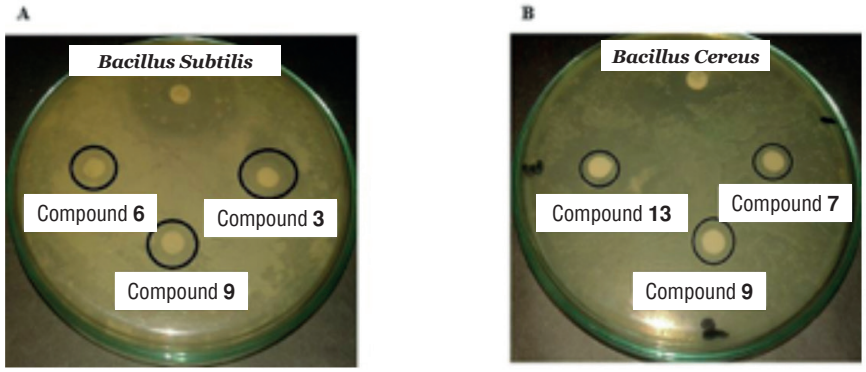


Figure 2. % Zone of inhibition of the compounds 3, 6 and 9 against *B. subtilis* (A) and the compounds 7, 9 and 13 against *B. cereus* (B).

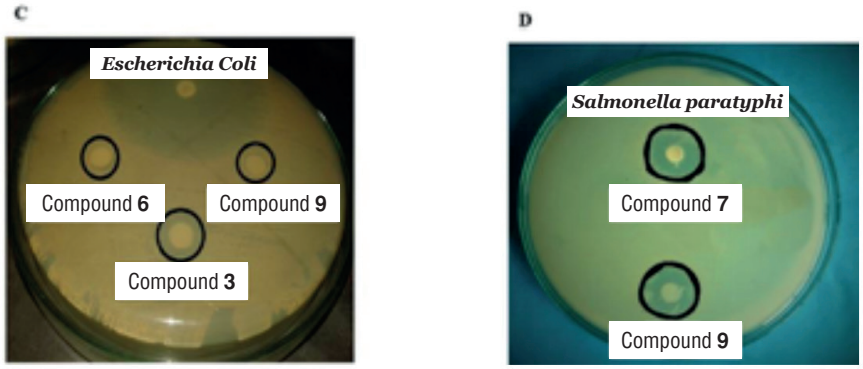


Figure 3. % Zone of inhibition of the compounds 3, 6 and 9 against *E. coli* (C) and the compounds 7 and 9 against *S. paratyphi* (D).

In general, it has been noticed that antibacterial results of the selectively acylated thymidine derivatives obtained by using various acylating agents follow the order for Gram positive organisms: **3 > 9 > 6 = 13 > 5 > 4 > 12 > 7 > 8** and Gram negative bacteria follow the order: **3 > 6 > 9 > 5 > 13 > 12 > 4 > 7.**

In a word this series of test chemicals was found to show very good antibacterial activity, particularly the presence of different acyl groups e.g. pentanoyl, decanoyl, benzenesulphonyl, 2-bromobenzoyl, cinnamoyl, groups improved the

antibacterial activity by a very good margin which was in accordance with our previous work[14]. We believe that a similar hydrophobic interaction might occur between the acyl chains of uridine accumulated in the lipid like nature of the bacteria membranes. As a consequence of their hydrophobic interaction, bacteria lose their membrane permeability, ultimately causing death of the bacteria[30-32].

CONCLUSION

We report an efficient one-pot synthesis of a small library of novel thymidine derivatives by direct method. We show that this one-pot three component reaction appears to be favorable for the preparation of variously substituted thymidine derivatives in moderate to good yields and opens the way for preparation of libraries of other nucleoside derivatives as potential biologically active molecules. Preliminary antibacterial in vitro screening against human pathogens showed that some of the prepared thymidine derivatives possess promising antibacterial activity. In addition, the antifungal, anticancer and antiviral activities of the thymidine derivatives thereof are currently under investigation and will be published in due course.

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Antioxidant, alpha-amylase and alpha-glucosidase inhibitory activities of leaf and flower extracts and fractions of *Phaulopsis falcisepala* C. B. Clarke

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Background: The methanol extracts of leaf and flower of *P. falcisepala* (PFL & PFF) were evaluated for inhibitors of alpha amylase/glucosidase (AGIs) with antioxidant property.

Methods: The total phenolic (TPC)/flavonoid content (TFC), antioxidant and digestive enzymes inhibitory activities of the PFL, PFF and PFF fractions; n-hexane (PFFHex), ethyl acetate (PFFEA), n-butanol (PFFBUT) and aqueous (PFFAQ) were evaluated.

Results: The PFF had higher TPC, TFC, greater antioxidant and digestive enzymes inhibitory activities than PFL. Thus, PFF fractions were further evaluated. The PFFEA had the highest TPC (161.05 ± 6.18 mgGAE/g), TFC (112.54 ± 1.69 mgQE/g), alpha amylase inhibitory activity (38.45 ± 2.50 µg/mL) than acarbose (258.4 ± 6.37 µg/mL $p < 0.05$). In addition, PFFEA showed higher activities in the Fe2+ reducing antioxidant power and alpha glucosidase inhibitory assays.

Conclusion: The promising activity of the flower of *P. falcisepala* can be further explored in the management of hyperglycaemia and oxidative stress.

Keywords: Alpha amylase, Alpha glucosidase, FRAP, DPPH, *Phaulopsis falcisepala*

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of the endocrine system characterized by hyperglycaemia due to defects in insulin secretion, insulin action or both[1]. The disorder impacts significant morbidity and mortality due to associated micro-vascular and macrovascular complications[2]. About 425 million people are living with diabetes worldwide[3]. Hyperglycaemia-induced oxidative and

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nitrosative stress have been singled out as the major links between diabetes and diabetic complications[4]. Hyperglycaemia leads to the generation of free radicals due to autoxidation of glucose and glycosylation of proteins[5]. Oxidative stress can cause endothelial dysfunction, insulin resistance and alterations in number and functions of pancreatic β-cells. Uncontrolled progression of diabetes and oxidative stress can eventually lead to diabetic microvascular and macrovascular complications[6]. Inhibition of enzymes involved in the metabolism of carbohydrate is one of the therapeutic approaches for reducing post-prandial hyperglycemia[7]. Inhibitors of amylase and glucosidase (AGIs) delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose level[8]. Clinically used AGIs include acarbose, emiglitate, miglitol and voglibose, they are used as adjunct to diet and exercise in Type II DM patients in whom glycaemic control is not achieved. Hypoglycaemia is not often observed with AGIs because they do not increase insulin secretion; however, increased delivery of carbohydrate to the colon is commonly observed, which results in increased gas production and gastrointestinal symptoms, such as flatulence and diarrhoea[9]. New AGIs are required to improve the efficacy and safety of this class of drugs. Effort in this study is to search for new AGIs with antioxidant property that can reduce hyperglycaemia-induced oxidative and nitrosative stress through ethnomedicine. Plants have been used since antiquity as source of drugs due to their high efficacy, reduced cost and minimal side effects[10]. Plant contains secondary metabolites that inhibit alpha-amylase which is one of the defence strategies against insect pest[11]. Also, the inhibitors of this enzyme are not readily available in the pharmaceutical industries[12]; this underscores the continuous search for inhibitors from plants. *Phaulopsis falcisepala* C. B. Clarke (Acanthaceae) is an erect or decumbent herb or shrub found throughout the forest zones from Senegal to Southern Nigeria. It is locally known among the Yorubas as *Opa-ogbe* and has been used by local indigenous groups for a wide range of medicinal purposes such as treatment of fever, rheumatic pain, diabetes, wounds, parasitic and fungal infection[13]. This study reports the antioxidant, alpha amylase and glucosidase inhibitory activities of *Phaulopsis falcisepala*.

METHODOLOGY

Chemicals and reagents

All reagents were purchased from Sigma Aldrich Co. St Louis, USA. 2, 2-Diphenyl-1- picryl- hydrazyl (DPPH), Aluminium chloride, trichloroacetic acid, Folin-ciocalteu's reagent, catechin, gallic acid, potassium ferricyanide, iron chloride, Acarbose, Starch, maltose, alpha amylase enzyme, alpha glucosidase enzyme, sodium potassium tartarate tetrahydrate, Dinitrosalicylic acid (DNSA),

Para nitro phenyl hydrazine (PNPG), Sodium bicarbonate distilled water, methanol, buffer salts (Sodium hydroxide, di-sodium hydrogen orthophosphate dehydrate and Sodium dihydrogen orthophosphate).

Plants collection and authentication

The leaf and flower of *P. falcisepala* (PFL & PFF) were collected in Ologun Eru area, Ibadan, Oyo State in September 2013. *P. falcisepala* was identified and authenticated by Mr. Chuckuma Emmanuel at the Forest Herbarium Ibadan, Forestry Research Institute of Nigeria (FHI 111021), Ibadan where voucher specimen was deposited.

Plant Extraction and Fractionation

The PFL and PFF (200 g) were air-dried and pulverized. They were extracted in 70% methanol by maceration at room temperature (29°C) for 72 hours. The extracts were filtered, and solvent removed using a rotatory evaporator. Percentage yields of the resulting methanol extracts were calculated. The methanol extract of PFF was dissolved in distilled water, partitioned into n-hexane (PFFHex), ethyl acetate (PFFEA), n-butanol (PFFBUT) fractions and the residue from the partitioning was aqueous fraction (PFFAQ). The fractions collected were concentrated with a rotatory evaporator. Percentage yields calculated, and fractions stored at 4°C till needed for further study.

***In vitro* antioxidant assay**

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

A modified method of Silva and co-workers was used to determine the scavenging activity of the plant extracts and fractions in DPPH based assay[14]. Briefly, gradient concentrations of the plant extracts and fractions (6.25 - 400 µg/mL) or standard drug ascorbic acid (0.25 - 16 µg/mL) were prepared in a 96-well plate. Test plates were incubated for 30 minutes in the dark at room temperature with freshly prepared solution of DPPH (0.04 mg/mL). Thereafter absorbance was read at 517 nm. Optical density (OD) measured was expressed as the percentage of the control.

Fe^{2+} reducing antioxidant power potential assay

The method of Hemalatha et al., 2010 was employed[15]. 1mL of varying concentrations of the extracts, fractions and ascorbic acid was added to 2.5mL of 0.2M phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixture was incubated at 50°C inside the water bath for 20 minutes. Furthermore, 2.5 mL of 10% trichloroacetic acid was added and the resulting mixture was centrifuged at 3000 rpm for 10 minutes. To 2.5mL of the clear supernatant, 2.5mL

of distilled water and 0.5mL of freshly prepared 0.1% iron chloride was added. The absorbance was read at 700 nm. A blank was prepared without the extract.

Determination of total phenolic content

The spectrophotometric method of Singleton et al., 1999 was used to determine the total phenolic content of the plant extracts and the fractions [16]. To 0.5 mL of the extract (1mg/mL), 2.5 mL of 10 % Folin-ciocalteu's reagent was added, followed by the addition of 2.5mL of 7.5 % sodium bicarbonate. The reaction mixture was then incubated at 45°C for 45 minutes and the absorbance was read at 765 nm. The same procedure was repeated for the gradient concentration of quercetin (0.125-1 mg/mL) prepared in methanol which was used to construct the standard curve. The concentration of the phenols in each sample was obtained from the calibrated curve and expressed in terms of quercetin equivalent (mg of QE/g of extract).

Determination of flavonoid concentration

The Aluminum chloride assay adopted from Quettier et al. (2000) was used in the spectrophotometric determination of the flavonoid present in the plant extracts and fractions[17]. 1 mL of 10 % of aluminum chloride was added to 1 mL of the extract/fractions (1mg/mL), the reaction mixture was further incubated at 25°C for 60 minutes. The absorbance was then read at 415 nm. The same procedure was repeated for varying concentrations (0.625 - 5 mg/mL) of gallic acid used to obtain the standard curve. The concentration of the flavonoid was obtained from the calibrated curve and then expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

***In vitro* digestive enzymes inhibitory activity**

Alpha amylase inhibition assay

A previously reported method of Tundis et al. (2010) was used to determine the inhibition of alpha amylase activity of the plant extracts and fractions[18]. Seven graded concentrations of the extracts, fractions and acarbose (31.25 - 1667 µg/mL) were obtained through two-fold serial dilution in phosphate buffer (pH 6.9). One milliter (1mL) of the extracts/fractions/acarbose was dispensed into well-labeled test tubes, and 1mL of 2 units of α- amylase solution was added. The resulting solution was pre-incubated for 15 min at 35˚C and the reaction initiated by the addition of 1mL starch solution (1%). The reaction mixture was further incubated for 5 min at 35˚C. Finally, 1mL of the colour reagent (96 mM 3, 5-dinitrosalicylic acid and 5.31 M sodium potassium tartrate in 2M sodium hydroxide) was added to terminate the reaction and the tubes incubated inside the water bath at 80°C for 15 minutes. The test tubes were removed, cooled on

ice and the reaction mixture was diluted with 9 mL of distilled water. Two hundred microliter (200 µL) each of the content in the test tubes was transferred into a microtitre plate and the absorbance was read at 540 nm. The amount of maltose formed was extrapolated from a maltose standard curve.

Alpha glucosidase inhibition assay

A previously reported method was adopted for use[19]. The plant extracts, fractions and acarbose were prepared in phosphate buffer (pH 6.9). Seven graded concentrations of the plant extracts/fractions/acarbose (6.25 - 800 µg/mL) were obtained by two-fold serial dilution in labeled test tubes. One milliter (1mL) of 1 U/mL of alpha glucosidase enzyme solution was pre-incubated with 500 µL of the plant extracts, fractions or acarbose in the test tubes for 10 min at 35˚C. Thereafter 500 µL of 3 mM of para-nitrophenylglucopyranoside (PNPG) was dispensed into the test tubes. The reaction mixture was then incubated for 15 mins at 35˚C. The reaction was terminated by the addition of 2 mL of 0.1M sodium bicarbonate. Two hundred microliter (200 µL) each of the content in the test tubes was dispensed into a microtitre plate and α-glucosidase activity was determined by measuring the amount of the yellow para-nitrophenol released from the PNPG at 405 nm in Spectramax Gemini XS microplate reader. In the two enzyme assays, control tubes were included in the assay tubes namely; coloured control tubes (contained serially diluted plant extracts/fractions or acarbose alone) and negative control tubes (i. e 100% enzyme activity, done by replacing plant extracts with buffer). The control tubes were also processed the same way as the test tubes.

Statistical analysis

All experiments were performed in triplicates and repeated in three independent experiments. Optical density (OD) was expressed as the percentage of the control. Percentage scavenging activity or inhibition of enzyme activity of the plant extracts, fractions or the standard drug in respect to the negative control was calculated. The 50% inhibitory concentration of plant extracts, fractions, ascorbic acid or acarbose (IC_{50}) was determined using non-linear regression in a commercial Mircocal Origin® statistical package. The IC_{50} values were means ± standard error of three independent data. Mann-Whitney U test was used to compare the mean IC_{50} of the plant extracts/fractions with that of the standard drugs. P-value < 0.05 was considered significant.

RESULTS

Yield of Plant Extracts

The percentage yield of the methanol extracts of the leaf and flower of *P. falcisepala* (PFL and PFF) were 5.5 and 3.8 % respectively (Table 1). Aqueous frac-

tion of P. *falcisepala flower* (PFFAQ) had the highest percentage yield of 66.3% among the fractions (Table 1).

Table 1. Percentage yield, total phenolic and flavonoid content of the methanol extracts and fractions of *P. falcisepala* leaf and flower

Plant extract/ fractions and drugs	Percentage Yield (%)	TPC (mgGAE/g)	TFC (mgQE/g)
P. falcisepala leaf (PFL) MeOH extract	5.5	19.53 ± 0.70	55.55 ± 0.58
P. falcisepala flower (PFF) MeOH extract	3.8	78.97 ± 1.13	77.22 ± 1.06
n-Hexane fraction (PFFHex)	8.6	9.51 ± 2.34	81.55 ± 0.77
Ethyl acetate fraction (PFFEA)	9.1	161.05 ± 6.18*	112.54 ± 1.69*
n-Butanol fraction (PFFBUT)	16.0	78.29 ± 3.82	19.42 ± 0.90
Aqueous fraction (PFFAQ)	66.3	20.99 ± 2.16	9.50 ± 0.51

*Ethyl acetate fraction had TPC and TFC significantly higher than other extracts and fractions ($P<0.05$).

Determination of Total phenolic and total flavonoid content

The total phenolic content (TPC) of the plant extracts/fractions expressed as mg of GAE/g of extract was obtained from the calibrated standard curve (equation $y= 0.100x + 0.013$, $r^2= 0.999$). The TPC of PFL and PFF were 19.53 ± 0.70 and 78.97 ± 1.13 mgGAE/g respectively (Table 1). In addition, the TPC in PFF fractions ranged from 9.51 ± 2.34 to 161.05 ± 6.18 mgGAE/g. The PFF fractions; PFFEA followed by PFFBUT had the highest TPC (Table 1). The total flavonoid content (TFC) values were expressed as mg of QE/g of the extracts/fractions as obtained from the standard curve with equation $y= 0.685x - 0.082$, $r^2= 0.988$. The TFC of PFL and PFF were 55.55 ± 0.58 and 77.22 ± 1.06 mgQE/g respectively (Table1). In addition, the TFC of PFF fractions ranged from 9.50 ± 0.51 to 112.54 ± 1.69 mgGAE/g. The fraction of PFF; PFFEA followed by PFFHex had the highest TFC (Table 1).

In vitro antioxidant activity of the methanol extracts and fractions of *P. falcisepala* leaf and flower

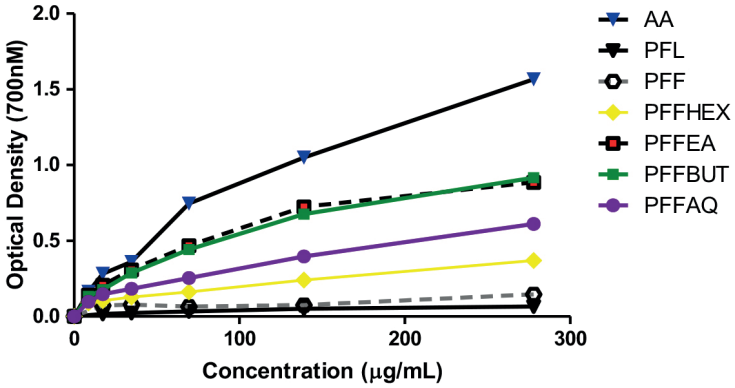
The IC50 of PFL and PFF in DPPH free radical scavenging activity are presented in Table 2. The PFL is inactive with IC_{50}> 400 µg/mL while PFF had IC_{50} of

28.76 ± 0.73 µg/mL (Table 2). The active PFF was dissolved in distilled water and partitioned into n-hexane, ethyl acetate and n-butanol yielding 4 fractions namely; PFFHex, PFFEA, PFFBUT and PFFAQ. Of the 4 fractions evaluated PFFAQ showed the highest antioxidant activity, followed by PFFHex with IC_{50} of 13.81 ± 0.77 µg/mL and 35.56 ± 1.34 µg/mL respectively (Table 2). Ascorbic acid the standard drug had IC_{50} of 7.87 ± 0.40 µg/mL. In the Fe^{2+} reducing antioxidant power, increase in absorbance value as the concentration increases shows increase in the reducing power of the extracts/ascorbic acid. Methanol extract of *P. falcisepala* flower is more active than its methanol leaf extract (Fig. 1). In addition, the order of antioxidant activity of PFF fractions is PFFEA > PFFBUT > PFFAQ > PFFHex (Fig. 1). At the highest concentration tested (277.77 µg/mL), the absorbance of PFF fractions ranged from 0.37 - 0.92 nm. The ascorbic acid had the highest absorbance (1.57 nm) followed by PFFEA and PFFBUT (0.89 & 0.92 nm).

Table 2. Alpha amylase and glucosidase inhibitory and DPPH scavenging activities of the methanol extracts and fractions of *P. falcisepala* leaf and flower

Plant extracts/ fractions and standard drug	50% Inhibitory Concentration (IC_{50}) µg/mL		
	Alpha amylase	Alpha glucosidase	DPPH Scavenging activity
P. falcisepala leaf (PFL) MeOH extract	880.0 ± 0.25	133.1 ± 3.24	> 400
P. falcisepala flower (PFF) MeOH extract	462.4 ± 2.53	86.05 ± 2.41	28.76 ± 0.73
n-Hexane fraction (PFFHex)	39.93 ± 3.28*	228.70 ± 1.70	35.56 ± 1.34
Ethyl acetate fraction (PFFEA)	38.45 ± 2.50*	52.99 ± 1.07**	83.77 ± 5.31
n-Butanol fraction (PFFBUT)	69.92 ± 8.60*	291.60 ± 1.77	180.10 ± 1.51
Aqueous fraction (PFFAQ)	38.65 ± 2.13*	154.90 ± 1.63	13.81 ± 0.77
Acarbose	258.40 ± 6.37	48.50 ± 1.30**	
Ascorbic Acid			7.87 ± 0.40

*All PFF fractions significantly active than acarbose (P<0.0001), **Both results were Comparable (P>0.06).



AA – ascorbic acid, PFL – methanol extract of P. falcisepalaleaf, PFF -methanol extract of *P. falcisepala* flower, PFFHEX – hexane fraction of P. falcisepala flower, PFFEA- ethyl acetate fraction of *P. falcisepala* flower, PFFBUT- buthanol fraction of P. falcisepala flower, PFFAQ-Aqueous fraction of *P. falcisepala* flower.

Figure 1. Fe^{2+} reducing antioxidant power potential of methanol extracts and fractions of *P. falcisepala* leaf and flower

In-vitro alpha amylase and glucosidase inhibitory activity of methanol extracts and fractions of P. falcisepala leaf and flower

The PFF produced a higher inhibitory activity against alpha amylase with IC_{50} of 462.40 ± 2.53 µg/mL while PFL had IC_{50} of 880.0 ± 0.25 µg/mL (Table 2). All the PFF fractions had greater activity (38.45 ± 2.50 - 69.92 ± 8.6 µg/mL) than acarbose (258.40 ± 6.37 µg/mL). Furthermore, in the alpha glucosidase inhibitory assay, PFF had higher inhibitory activity with IC_{50} of 86.05 ± 2.41 µg/mL than PFL (IC_{50} of 133.1 ± 3.24 µg/mL, Table 2). In addition, the PFFEA was the most active with IC_{50} of 52.99 ± 1.07 µg/mL (Table 2). The IC_{50} value of the standard drug acarbose was 48.50 ± 1.30 µg/mL.

DISCUSSION

Diabetes is a metabolic disease that is associated with chronic hyperglycemia as a result of disturbance in carbohydrate, fat and protein metabolism due to defects in insulin secretion and action[1]. Uncontrolled hyperglycemia can cause excessive generation of intracellular reactive oxygen species (ROS), thus leading to oxidation of important macromolecules including proteins, lipids, carbohydrate and DNA[5]. Inhibition of enzymes involved in the metabolism of carbohydrate is one of the therapeutic approaches for reducing post-prandial hyperglycemia[8]. Thus, the approach in this study is to evaluate *Phaulopsis falcisepala* for inhibitors of α-amylase and α-glucosidase with antioxidant activity.

This is because plants have been shown to produce a large variety of amylase inhibitors that offer protection against insects, their larvae, and microbial pathogens[11]. Previous antioxidant report showed that *P. fascisepal*a leaf extract had less scavenging effect on DPPH radical and reducing power on Fe^{3+}/ferricyanide complex but better Fe^{2+}-chelating ability[20]. However, in this study we evaluated both the leaf and flower of *P. fascisepala*. The flower of methanol extract of *P. falcisepal*a (PFF) was 13 times more active than its leaf extract in the DPPH radical scavenging assay. The PFF had higher phenols and flavonoids content than its leaf extract. The higher antioxidant activity observed in the PFF compared to PFL extract could be due to the abundant presence of major secondary metabolites such as phenols and flavonoids. Several studies have reported that polyphenols, such as flavonoids, hydroxycinnamic acids and proanthocyanidins, act as powerful antioxidants[21-23]. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities[24]. Phenolic antioxidants have been recognized as an important class of food ingredients and are currently added to various food products in order to provide additional health benefits[23, 25]. Furthermore, in the Ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP), increase in absorbance as the concentration increases is an indication of a strong reducing potential. Ethyl acetate (PFFEA) and butanol (PFFBUT) fractions of PFF showed stronger reducing potential than the other fractions and extracts. In the α-amylase and α-glucosidase inhibitory assays, PFF was more active in the two assays than PFL. Fractionation of PFF resulted in four fractions that were significantly more active than its crude extract in alpha amylase inhibitory assays. All the four fractions of PFF showed higher α-amylase inhibitory activity than acarbose ($p<0.001$). It appears its alpha amylase inhibitory activity is distributed in the four fractions unlike the DPPH and alpha glucosidase inhibitory activities that appear to be concentrated in the aqueous and ethyl acetate fractions, respectively. There are limited pharmacological studies on this plant. The alpha amylase and alpha glucosidase inhibitory activities of the flower of *P. falcisepala* appeared to be reported in this study for the first time.

CONCLUSION

The flower of *Phaulopsis falcisepala* showed higher antioxidant, alpha amylase and glucosidase inhibitory activities than its leaf. The activity observed might be due to higher phenolic and flavonoid contents in the flower than the leaf. The flower of *P. falcisepala* can serve as potential sources of natural agents that can be further explored to control post prandial hyperglycemia and associated oxidative stress.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Hypoglycemic Potential of Combined Ethanol Extracts of *Gongronema latifolia* and *Vernonia amygdalina Leaf* in Alloxan-induced Diabetic Albino Wistar Rats

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ABSTRACT

The effect of *Gongronema latifolia* (GL) and *Vernonia amygdalina* (VA) leaf extracts on glucose level in alloxan-induced diabetic rats was studied. Sixty albino rats (170g-200g) were divided into twelve groups with five animals per group. Group 1 and 12 served as normal and diabetic controls respectively. Groups 2, 3 and 4 were normal rats and received 150mg/kg of GL, VA and combined 100mg/kg each of GL and VA respectively. Groups 5, 6 and 7 were treated as Groups 2, 3 and 4 respectively before diabetes induction. Groups 8, 9 and 10 were treated as Groups 2, 3 and 4 respectively while Group 11 was treated with insulin after diabetes induction. Extract administration lasted for 14 days. Glucose level was significantly ($P < 0.01$) lowered in normal and post-diabetic treated rats as well as Groups 5 and 6. Combine extract of *Gongronema latifolia* and *Vernonia amygdalina* have good glucose lowering potential.

Keywords: *Vernonia amygdalina, Gongronema latifolia,* Diabetes, Blood Glucose, Insulin.

INTRODUCTION

Plants have been used in traditional medicine for the treatment of ailments for thousands of years and there is still increasing interest in the study of medicinal plants and their uses in different parts of the world.[1,2] Reports have stated that about 80% of the world's population still depend on traditional medicine for their primary health care and there are considerable economic benefits in the development of indigenous traditional remedies for the treatment of various disease[3].

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Diabetes is a multifaceted disease in terms of its pathogenicity and has been identified as a group of metabolic disorders with hyperglycemia, polynuria, polydipsia, polyphagia and glucosuria as the major characteristics[4]. The hyperglycemia is a manifestation resulting from several metabolic anomalies and depicts a complete breakdown in metabolic activities rather than a pathogenic factor[5]. The pathological scenario in diabetes presents multiple therapeutic targets that may require a range of different agents to address the different features of the disease in different stages of its development. Individual therapeutic agents act only on part of the pathogenic process and only to a partial extent hence cannot holistically address the problem[6]. This contributes significantly to the increased morbidity and mortality of diabetes despite the detailed understanding of the condition and the availability of several pharmacological agents for its management.

Several orthodox medications exist for the management of diabetes, however, evidence has also shown that various herbal therapy has been proven useful in the management of diabetes especially in the underdeveloped and developing world.[7] *Gongronema latifolia* and *Vernonia amygdalina* are medicinal plants frequently consumed in Nigeria for its nutritional and pharmacological effects.[8,9,10] The plants have been widely studied as their anti-bacterial, anti-diabetic, anti-fungal, anticytotoxic, anti-pyretic, anti-inflammatory and anti-oxidant potentials have been documented.[11,12,13] Phytochemical screening of *Vernonia amygdalina* and *Gongronema latifolia* have been reported and the plants are rich in polyphenols, tannins, flavonoids, terpenoids, saponins, alkaloids, cardiac glycosides as well as steroids.[14,15] These phytochemicals (singly or in synergy) are responsible for the pharmacological and medicinal effects attributed to these plants.

The search for appropriate agents in the management of diabetes is therefore focused on traditional medicinal plants due to leads that have been provided by traditional plants and the presence of several medicinal phytochemicals in plants that have the potential to act on variety of targets by various modes and mechanisms.[16,17] Moreover, the combination of various agents from different plant sources have been used to enhance the efficacy of polyherbal formulations. Polyherbal therapies have the synergistic, agonist/antagonist pharmacological properties that can promote maximum therapeutic efficacy with minimum adverse effects.[17]

The present study was aimed at evaluating the glucose lowering potential of combined *Gongronema latifoli*a and *Vernonia amygdalina* leaf extract in alloxan-induced albino Wistar rats in view of formulating a polyherbal therapy for the management of diabetes.

METHODOLOGY

Plant Materials

Fresh Leaves of *Gongronema latifolia* and *Vernonia amygdalina* were obtained from Uyo metropolis, Akwa Ibom State, Nigeria. The plants were authenticated by Dr (Mrs) Margaret E. Bassey, a taxonomist at the Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria. *Vernonia amygdalina* and *Gongronema latifolia* were assigned voucher number UUH2083 and UUH2084 respectively. The leaves were washed with clean water and air dried at room temperature. Separately, the leaves were blended into powdered form and were macerated in 80% ethanol for 48 hours. The filtrate from each extract was obtained and concentrated in a water bath at 45°C to obtain the crude extract of both leaves. The crude extracts were preserved in a refrigerator at -4°C until used.

Experimental Animal and Design

Sixty (60) albino rats weighing between 170 – 200 grams were used for the study and were divided into twelve (12) groups with five (5) animals in each group. The animals were obtained from the Department of Biochemistry Animal House, University of Calabar, Calabar, Nigeria. The rats were maintained under standard laboratory conditions and allowed access to food and clean water *ad libitum*. Group 1 and 12 served as normal and diabetic controls respectively. Groups 2, 3 and 4 received 150mg/kg of *Gongronema latifolia*, 150mg/kg of *Vernonia amygdalina* and combined 100mg/kg of *Gongronema latifolia* and 100 mg/kg of *Vernonia amygdalina* respectively without induction of diabetes. Groups 5, 6 and 7 received 150mg/kg of *Gongronema latifolia*, 150mg/kg of *Vernonia amygdalina* and combined 100mg/kg of *Gongronema latifolia* and 100 mg/kg of *Vernonia amygdalina* respectively before induction of diabetes. Groups 8, 9, 10 and 11 were treated with 150mg/kg of *Gongronema latifolia,* 150mg/kg of *Vernonia amygdalina*, combined 100mg/kg of *Gongronema latifolia* and 100 mg/kg of *Vernonia amygdalina* and insulin respectively after induction of diabetes. The extracts were orally administered daily for 14 days.

Induction of Diabetes with Alloxan

Alloxan selectively destroys the insulin-producing islet of Langerhans in the pancreas when administered to rodents resulting in insulin dependent diabetes mellitus which is similar to Type 1 diabetes in humans [18]. The body weight and blood glucose levels of the animals to be made diabetic were determined before the administration of alloxan to induce diabetes. Diabetes was induced by intraperitoneal administration of 150mg of alloxan per kilogram body weight of the experimental animal. After administration of alloxan, the animals were observed

for signs of diabetes which include polyuria, polydipsia, polyphagia and hyperglycemia. Blood sample was obtained through the tail of the experimental animals to check for blood glucose levels using a glucometer and glucose test strips as confirmatory test for hyperglycemia.

Determination of Blood Glucose Levels

At the end of the experiment, the animals were sacrificed under chloroform anesthesia. Blood sample was obtained through cardiac puncture and centrifuged at 3000rpm for 15 minutes to obtain the serum which was used for assay of glucose concentration. Randox-assay kit (GOD-PAP) method based on method described by Barham and Trinder was used for the assay.[19] The principle involves the enzymatic oxidation of glucose in sample by the enzyme glucose oxidase which generates hydrogen peroxide and gluconic acid. The concentration of H_2O_2 released is proportional to initial amount of glucose in the sample and it reacts under catalysis of peroxidase, with phenol and 4-amino phenazone to form a red violet quinoneimine dye whose colour intensity reflects the concentration of glucose in the sample.

Statistical Analysis

The data obtained were expressed as Mean ± SEM. SPSS software, Version 20.0 was used for statistical analysis of the data. One-way analysis of variance (ANOVA) was used for comparison and results were subject to post hoc test using Tukey multiple comparison tool. Test values of $p < 0.01$ were considered significant.

RESULTS

The result of the combined effect of *Gongronema latifolia* and *Vernonia amygdalina* on the blood glucose in the study is presented in Table 1. A significant decrease in the blood glucose level was observed in the non-diabetic rats administered the extracts when compared with the normal control. Similarly, a significant decrease in blood glucose levels was seen in post-diabetic treated groups compared to both the normal and diabetic control. Only the blood glucose of the *Gongronema latifolia* treated group in the pre-diabetic treated groups was significantly decreased when compared to the control.

Table 1. Effect of Combined Administration of *Gongronema latifolia* and *Vernonia amygdalina* on the Blood Glucose Level of Alloxan Induced Diabetic Rats.

GROUPS	BLOOD GLUCOSE (mg/dl)
Group 1 – Normal Control	60.60 ± 1.71
Group 2 – *Gongronema latifolia*	54.90 ± 1.79*
Group 3 – *Vernonia amygdalina*	50.60 ± 0.22*
Group 4 – *G. latifolia and V. amygdalina*	50.20 ± 0.86*

Group 5 - *Gongronema latifolia* before Alloxan	47.90 ± 0.57*#
Group 6 - *Vernonia amygdalina* before alloxan	58.30 ± 0.62#
Group 7 - *G. latifolia and V. amygdalina* before alloxan	69.00 ± 0.71*
Group 8 – Alloxan before *Gongronema latifolia*	57.40 ± 0.50*#
Group 9 – Alloxan before *Vernonia amygdalina*	41.30 ± 0.40*#
Group 10 – Alloxan before *G. latifolia and V. amygdalina*	40.20 ± 0.49*#
Group 11 – Alloxan before Insulin	60.80 ± 1.02#
Group 12 – Alloxan alone	68.80 ± 0.74*

*Data are presented as Mean ± Standard Error of Mean (SEM). Values are considered significantly different at p < 0.01. * = Significantly different from normal control (Group 1). # = significantly different from diabetic control (Group 12).*

DISCUSSION

Glucose concentration in the blood at any given time is determined by metabolic processes in the liver, muscle and adipose tissue which is under regulation by insulin and glucagon.[20] The target of diabetic treatment is to restore the normal metabolic processes and regulation resulting in normal glycemic level in the host. The administration of *Gongronema latifolia* and *Vernonia amygdalina* singly and in combination has been shown to demonstrate hypoglycemic result in normoglycemic rats. This is suggestive of the fact that the extracts may stimulate insulin production from the pancreatic β-cells or increase the activity of glucose transporters on target tissue membrane.[21]

However, when the extracts were administered prophylactically (pretreatment before inducing diabetes), the blood glucose of the *G. latifolia* and *V. amygdalina* treated groups tend towards that of the normal control. The combined extract treated group still had glucose level similar to the diabetic control group. This suggests that the combined extract had no protective effect against the destruction of insulin producing islet of Langerhans cells by alloxan. Alloxan selectively destroys the insulin producing islet of Langerhans in the pancreas when administered to rodents resulting in insulin dependent diabetes mellitus which is similar to Type 1 diabetes in humans.[18]

Mfon *et al.,* had earlier reported the effect of combined administration of *Vernonia amygdalina* and *Gongronema latifolia* on the pancreatic β-cells of streptozotocin induced diabetic rats[21]. They reported a regeneration and proliferation of islet cells of diabetic rats which were destroyed by streptozotocin during the induction of diabetes. The glucose lowering potential observed in the present

study may be due to the fact that there was regeneration of the islet cells that were destroyed by alloxan which was used to induce diabetes in the animals. Regeneration of pancreatic beta cells have been reported as a probable mechanism of hypoglycemic action of *Vernonia amygdalina*.[21] Regeneration of the islet cells restores the synthesis of insulin which then facilitate the uptake of glucose from the blood into the cells. Mfon *et al.,* also reported that the extracts of these plants have insulin mimetic effects in addition to regeneration of the islet of Langerhans which enhances endogenous insulin production.[22]

It is believed that phytochemicals such as tannins, flavonoids, glycosides and phytosterols present in *Vernonia amygdalina*[23] and alkaloids, glycosides and saponins present in *Gongronema latifolia*[24] may contribute to the hypoglycemic potential of the plants singly and in combination. Reports have shown that polyherbal therapies have the synergistic, agonist/antagonist pharmacological properties that can promote maximum therapeutic efficacy with minimum adverse effects.[17]

It can therefore be concluded that *Gongronema latifolia* and *Vernonia amygdalina* have good hypoglycemic potentials that is even better in combination in the treatment of hyperglycemia induced by alloxan in albino Wistar rats.

ETHICAL APPROVAL

Ethical approval for the study was obtained from the Research Ethical Committee of Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria.

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Antiprotozoal, antiviral and cytotoxic properties of the Nigerian Mushroom, Hypoxylon fuscum Pers. Fr. (Xylariaceae)

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ABSTRACT

In Nigeria, mushrooms are utilised for the treatment of several ailments. Although the biological activities of some Nigerian mushrooms have been reported, there is dearth of information on the therapeutic potentials of *Hypoxylon fuscum*. The aim of this study is to investigate the antileishmanial, antiplasmodial, antitrypanosomal, cytotoxic and antiviral activities of *H. fuscum*. The antiplasmodial studies revealed that the extract was active against chloroquine-sensitive D6 and chloroquine-resistant W2 strains of *Plasmodium falciparum* (IC_{50} of 6.98 and 8.33 µg/mL, respectively). The extract showed antitrypanosomal activity on *Trypanosoma brucei brucei* but lacked inhibitory activity against *Leishmania donovani*. The extract displayed cytotoxicity on *Artemia salina* larvae and rhabdomyosarcoma cell line, with CC_{50} value of 3.33 and 8.60 µg/mL, respectively, and also displayed antiviral activity on echoviruses (E7 and E19). This study demonstrated that *Hypoxylon fuscum* possess several pharmacological activities and may provide a drug lead for the development of effective chemotherapeutic agents.

Keywords: Antiprotozoal activity; Echoviruses; *Hypoxylon fuscum*; MTT assay; Mushroom

INTRODUCTION

In today's world, the ever-increasing global demand for natural products as a credible source of pharmaceutical products, has placed intense pressure on several angiosperms. In recent times, efforts are now being channelled toward the exploitation of lower organisms such as bacteria and fungi[1]. Mushrooms are fungi, that lack chlorophyll, and thus derive their nutrients from the metabolism

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of non-living organic matters, such as putrefying leaves and tree trunks. Of the 140,000 known species of mushrooms that exist globally, about 10% have been properly identified and only approximately 5% have been investigated for their pharmacological activities[2]. Mushrooms with medicinal properties (medicinal mushrooms; MMs) have been reported to possess more than 100 medicinal functions, with the notable ones being antineoplastic, antidiabetic, antiviral, antibacterial, antifungal, immunomodulatory and antiparasitic properties[3]. MMs are also used in the food industry as dietary foods and supplements; the cosmetic industry as cosmeceuticals; and in the agriculture sector as pesticides, herbicides and insecticides. Various pharmaceuticals with secondary metabolites obtained from MMs have made it to several clinical trials. For instance, the polysaccharide extract of Maitake mushroom, *Grifola frondosa,* showed excellent Phase II clinical trial results in breast cancer patients[4]. Krestin, a polysaccharide isolated from the fruiting body of *Trametes versicolor*, was reported to inhibit tumor growth in animal models and human clinical trials[5,6]. Other notable pharmaceuticals formulated from MMs available in global drug industry include lentinane, copsin and schyzophillan, which is used as antitumor, antimicrobial and immunomodulatory agent, respectively[7].

In Nigeria, mushrooms are utilised for the treatment of several ailments[8-10]. For example, the extracts of *Ganoderma lucidum* and *Ganoderma appalanatum* are used for the management of arthritis, diabetes and liver diseases in the southern part of Nigeria[11, 12]. *Pleurotus tuberregium* is utilised for the treatment of headache, fever, cold and constipation[13]. In addition, *Lycoperdon umbrinum* (puffball) is used in southwestern Nigeria for treating wounds, inflammation, diarrhoea and other ailments[14].

Hypoxylon fuscum Pers. Fr., like several other members of the Xylariaceae family, grows on dead trees, especially on the trunk. Xylariaceous fungi, especially the genus *Hypoxylon*, have been shown to contain diverse bioactive secondary metabolites[15], including antimicrobial azaphilones from *H. multiforme*[16], carneic acids A and B from *H. carneum*[17], sclerin and its diacid from *H. fragiforme*[18]. While there have been several reports of biological activities of other *Hypoxylon species*[19-21], a thorough search of available literature revealed that the therapeutic potentials of *H. fuscum* remains largely unexplored. Therefore, this work was carried out to investigate the phytochemical constituents, as well as, the antiplasmodial, antileishmanial, antitrypanosomal, antiviral and cytotoxic activities of the methanol extracts of *H. fuscum* in several in vitro models.

METHODOLOGY

Extraction of mushroom

Fresh samples of the mushroom, *Hypoxylon fuscum*, were collected from its natural habitat (dead branches of several trees) in the premises of the University of Ibadan, Ibadan, Nigeria, between June and September 2017, and identified by Mrs Jumoke Morounfolu of the Department of Botany, University of Ibadan, Nigeria. The air-dried and pulverised mushroom (1.1 kg) was extracted into MeOH (4 L) at room temperature (25-32 °C) for a 72-h period. The crude extract obtained was concentrated using a rotary evaporator at 40 °C and stored in a refrigerator at 4 °C, prior to use.

Phytochemical screening

Following standard procedures described in earlier literature[22,23], the phytochemical analysis of the mushroom extract was carried out to determine the presence of several secondary metabolites, including alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, sterols and tannins.

Antiplasmodial assay

Antiplasmodial assay was carried out at the National Centre for Natural Products Research, University of Mississippi, USA. In this assay, the parasitic lactate dehydrogenase (pLDH) activity was estimated as a measure of the antiplasmodial potential of the extract[24,25]. Briefly, erythrocytes infected with chloroquine-sensitive (D6) or chloroquine-resistant (W2) strains of *Plasmodium falciparum* (2% parasitemia and 2% hematocrit) were prepared in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% human serum and 60 μg/mL amikacin. 200 μL of the preparation was added into each well of a 96-well microplate containing 10 μL of various concentrations of the extract. The positive controls included artemisinin and chloroquine (obtained from Sigma Chemicals Co.), while DMSO contained medium served as the negative control. For the primary antiplasmodial assay, the extract was tested at a single concentration of 15.9 μg/mL, while test concentrations ranging from 5.3 to 47.6 μg/mL was used for the secondary antiplasmodial assay. The experiment was carried out in triplicate and the IC_{50} values calculated from a dose-response curve obtained from the GraphPad software.

Antileishmanial assay

The alamar blue assay was performed on a culture of *Leishmania donovani* promastigotes and axenic amastigotes to determine the antileishmanial potential of the extract[26]. Optimum growth conditions were ensured for the parasites before

treatment was commenced. The promastigotes were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and maintained at pH 7.4 in a humidified atmosphere of 5% CO2 at 26 °C. The axenic amastigotes were cultured in RPMI medium supplemented with 4-morpholineethanesulfonic acid (MES) (4.88 g/L), L-glutamine (298.2 mg/L), adenosine (26.7 mg/L), folic acid (10.1 mg/L), BME vitamin mix, sodium bicarbonate (352.8 mg/L) and 10% FBS and maintained at 37 °C and 5% CO2, with the pH of the medium fixed at 5.5. In the primary assay, the parasites were treated with a single concentration (20 μg/mL) of the extract and incubated for 72 h at 26 °C and 37 °C, respectively, for promastigotes and axenic amastigotes. No further secondary assay was carried out as the extract displayed little or no activity in the primary assay.

Antitrypanosomal assay

For this test, a modification of a method described recently[27] was used. In brief, two days culture of *Trypanosoma brucei brucei* was diluted in a 96-well microplates, containing Iscove's Modified Dulbecco's medium (IMDM) to obtain 5000 parasites/mL. The culture was maintained in a humidified atmosphere of 5% CO2 at 37 °C. For the primary antitrypanosomal assay, 4 μL of the extract (at a single concentration of 20 μg/mL) was added into each well of the microplate containing 196 μL of the IMDM-parasite culture to make a final culture volume of 200 μL. The plates were incubated at 37 °C in 5% CO2 for 48 h. Thereafter, 10 μL of alamar blue (AbD Serotec) was incorporated into each well and the plates were further incubated for 24 h. At the end of the incubation period, the fluorescence was measuered on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation and 590 nm emission. For the secondary screening, the procedure described above was repeated, only that the test concentration ranged from 10 to 0.4 μg/mL. The IC_{50} and IC_{90} values were estimated from the dose-response analysis curve obtained from XLfit version 5.2.2.

Cytotoxicity screening

Brine Shrimp Lethality Assay (BSLA)

The BSLA is a simple, unsophisticated, bench-top assay used to identify extracts with potential cytotoxic compounds. The protocol described by McLaughlin, 1991, with little modification, was followed in this work[28]. In brief, *Artemia salina* (brine shrimp) eggs were hatched in a vessel filled with natural sea water under constant aeration for 48 h. After hatching, a Pasteur pipette was used to collect ten nauplii into several tubes containing 4.5 mL of brine solution. The extract was serially diluted to obtain working concentrations ranging from 1000 to 1 μg/mL. 0.5 mL of each concentration was added to the vial containing the ten nauplii

and the brine solution and incubated for 24 h at room temperature (25 – 32 °C). The number of dead and live nauplii in each well was counted using a magnifying lens. Cyclophosphamide was used as the positive control while brine solution alone was used as the negative control. The experiment was carried out in triplicate at room temperature (25 – 32 °C). The concentration responsible for killing 50% of the nauplii population (LD_{50}) was calculated using the GraphPad Prism software.

Cell culture and virus

The RD cells, obtained from the Centre for Disease Control, Atlanta, were cultured in T25 flask containing Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and1% penicillin-streptomycin antibiotics. Cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C. Cells were passaged in ratio 1:3 once it reaches confluency following treatment with 0.05% trypsin. Cells were viewed using an inverted microscope. Three serotypes of echovirus (E7, E13, and E19) isolated from stool at the WHO Polio Laboratory, Department of Virology, University of Ibadan, Nigeria, were used for the antiviral studies. The viruses were stored at –70 °C until use.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) cell viability assay

The MTT assay was carried out to determine the effect of the *H. fuscum* extract on the viability of rhabdomyosarcoma (RD) cells. The method described earlier by Mosmann, 1983, was followed with slight adjustments[29]. Briefly, RD cells suspension (100 µL) were seeded into 96-well plates and maintained in the incubator for 24 h. Subsequently, the cells, which obtained a confluency of about 90%, were treated with serial dilutions (100 µL each) of the mushroom extract, ranging from 1000 – 0.01 µg/mL. The extract itself was previously dissolved in DMSO and diluted with the culture medium, ensuring the maximum concentration of DMSO was not more than 0.05%, a concentration that is not toxic to cancer cells[30]. The treatment was carried out for a period of 72 h in the same environment mentioned above. Following this, the old culture medium was removed, and the cells monolayers were washed with phosphate buffer saline (PBS). Thereafter, 100 µL of MTT solution (500 µg/mL) was added into each well of the 96 well plates and incubated at 37 °C for 2 hours. After the incubation period, the preformed formazan crystals were solubilised using DMSO and the optical density (OD) was measured at 570 nm using a multiplate reader (Thermoscientific, Waltham, MA). Cyclophosphamide was used as the negative control while growth medium was used as the negative medium. The experiments were carried out in triplicate with the OD of the negative control fixed arbitrarily

at 100%, and OD of treatments calculated relative to negative control. The concentration responsible for reducing the viability of the cells by 50% (CC_{50}) was calculated using a non-linear regression curve generated from the GraphPad Prism 5 software (GraphPad, USA).

50% tissue culture infective dose ($TCID_{50}$)

To determine the 50% tissue culture infective concentration ($TCID_{50}$), the Sperman-Karber's method was used to estimate the virus titres that caused cytopathic effect (CPE) in RD cell culture. In brief, 100 µL of E7 were added into a T25 culture flask (Corning®, UK), containing RD cells and incubated for 72 h. This increased the virus stock quantity due to the 100% CPE.

Thereafter, 100 µL RD cells (1 x 106 cells/mL) were seeded into three 96-well plates and incubated for 24 h. The virus stock was diluted serially in ten-fold and 100 µL of each dilution was inoculated into the wells. The cell control used contained RD cells without the virus. The plates were incubated at 37 °C and daily CPE scoring was done till the control wells started dying. The 100 $TCID_{50}$ was used for the antiviral assay and the procedure was repeated for E13 and E19.

Antiviral assay

The neutralisation test[31], an assay that measures CPE inhibition in cell culture was used to investigate the antiviral activity of *H. fuscum* extract against echoviruses (E7, E13, and E19). Briefly, 50 µL of 100 $TCID_{50}$ virus suspension were added into a 96-well plate containing a monolayer of confluent RD cells and allowed to stand for 1 h to enable virus adsorption. Subsequently, ten-fold serial dilutions of the extract, starting from the maximum non-toxic concentration (10 µg/mL), was added in triplicate into the wells, except for the negative control and virus control wells that contained only the RD cells and virus, respectively. The plates were incubated at 37 °C in 5% CO2 humidified incubator for 72 h after which the cell viability was measured using the MTT assay as described above. The 50% inhibition concentration (IC_{50}) was defined as the concentration that reduces CPE by half, with respect to the virus control. The selectivity index (SI) was calculated as the ratio of the extract's concentration that reduces viability of the RD cells by 50% (CC_{50}) to the concentration of the extract that inhibit cytopathic effect to 50% of the control value (IC_{50}). Since there are no antiviral drugs approved for the treatment of enteroviral infections, no drug control was used in this study.

High performance liquid chromatography (HPLC) analysis

The Dionex HPLC system 2695 (Waters) coupled with a Thermoscientific NX 5 µm C18 column (250 x 4.6 mm) and a library of chemical compounds were used

for this analysis. For the experiment, column temperature and variable ultraviolet-visible detector were maintained at 25 °C and 235 nm, repsectively. The binary solvent system comprising of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in methanol (solvent B) was used as the eluent. Reversed-phase analytical HPLC analysis was done as follows; 0-40 min: 30-100% B; 41-50 min: 100% B; 51-60 min: 100-30% B; flow rate, 1 mL/min. Determination of the compounds present in the extract was carried out by comparing the UV of the peaks obtained in the HPLC chromatogram with compounds collection contained in the HPLC library.

RESULTS AND DISCUSSION

The ever-expanding exploitation of medicinal plants as a tool for boiactive compounds discovery have stimulated the interest of natural product scientists in the discovery of potent pharmaceutical prodcuts from medicinal mushroom (MMs). MMs represent a huge but widely unexploited source of new chemotherapeutic agents. In this study, the biological activities of a Nigerian mushroom, *Hypoxylon fuscum*, was investigated. The qualitative analysis of the phytochemical constituents of *H. fuscum* revealed that it contained several secondary metabolites including alkaloids, cardiac glycosides, coumarins, flavonoids and sterols. However, anthraquinones, saponins and tannins were absent in the extract (Table 1).

Table 1. Phytochemical constituents of H. fuscum MeOH extract

Phytochemicals	Results
Alkaloids	++
Anthraquinones	-
Cardiac glycosides	+
Coumarins	++
Flavonoids	++
Saponins	-
Sterols	++
Tannins	-

Key: - = absent; + = present; ++ = abundant

Malaria remains a major threat to global health as the populace of most tropical and subtropical nations of the world are faced with this disease. In addition, recent studies have reported parasitic resistance to currently used antimalarial agents[32,33]. Since natural products have played significant role in the discovery of antimalarial molecules, as exemplified in the discovery of artemisinin from *Artemia annua* and quinine from *Cinchona succirubra*, it is needful to further

explore natural sources to discover new antimalarial leads. The *in vitro* antiplasmodial investigation revealed that *H. fuscum* extract was potent against both chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum* with IC_{50} values of 6.98 and 8.33 µg/mL, respectively, when compared to chloroquine and artemisinin, that displayed IC50 values less than 0.0264 µg/mL against both strains of malaria parasites (Table 2).

Table 2. Antiplasmodial activity of *H. fuscum*

Extract/ Compound	Primary (% inhibition)	Secondary (IC_{50}; µg/mL)		
	D6	W2	D6	W2
H. fuscum	74	68	6.98	8.33
Chloroquine	ND	ND	<0.0264	<0.163
Artemisinin	ND	ND	<0.0264	<0.0264

ND: not determined; IC_{50}: 50% inhibition concentration

Previous reports on antiplasmodial screening of crude extracts have stated that extracts with IC_{50} value less than 10 µg/mL possess high activity, IC_{50} between 10 µg/mL and 50 mg/mL possess moderate activity and those with IC_{50} greater than 50 µg/mL possess no activity[34,35]. It is therefore safe to state that the extracts of *H. fuscum* possess bioactive molecules with potent antiplasmodial activity.

The extract exerted a significant antitrypanosomal activity on *Trypanosoma brucei brucei* with IC_{50} value of 10.79 µg/mL. *T. brucei brucei* is a pathogenic protozoan that causes human African Trypanosomiasis (HAT; sleeping sickness) and animal trypanosomiasis in humans and animals, respectively[36]. HAT is a neglected tropical disease that is assocaited with high mortality, especially when mangement is not insituted early. The scarcity of effective chemotherapy for the treatment of this disease and the increase in the resistance of parasite towards available drugs have necessitated the search for the discovery and development of more potent bioactive molecules for the management of HAT[37]. With the potent antitrypanosomal acitivity displayed by the extract of *H. fuscum,* especially its 77% inhibition of the parasite in the primary assay (Table 3), this mushroom could provide a template for the discovery of agents with antitrypanosomiasis activity. However, the extract lacked significant inhibitory activities on the both promastigotes and amastigotes of *L. donovani*, as it displayed IC_{50} greater than 20 µg/mL in the secondary assay.

Table 3. Antileishmanial and antitrypanosomal activity of *H. fuscum*

Parasite	Primary (% inhibition)	Secondary	
		(IC50; µg/mL)	(IC90; µg/mL)
L. donovani promastigotes	0	>20	>20
L. donovani amastigotes	46	>20	>20
T. brucei brucei	77	10.79	> 20

IC_{50}: 50% inhibition concentration; IC_{90}: 90% inhibition concentration

To determine the cytotoxic properties of H. fuscum extract, the brine shrimp lethality assay (BSLA) and the MTT viability assay were used. The BSLA is an assay used in the preliminary screening of plant extracts for the presence of chemotherapeutic molecules. Some studies have reported that plants with toxicity toward Artemia salina larvae may have potentials in anticancer drug discovery[38,39]. The MTT assay is a colorimetric assay that measure cell viability, with respect to the ability of mitochondrial reductase present in viable cells, to reduce water soluble MTT to water insoluble formazan crystals. The quantity of formazan produced at the end of the assay is used to estimate the amount of viable cells in the culture medium[40]. The National Cancer Institute (NCI) has stated that a crude extract should be considered active in a preliminary cell viability studies if it as CC50 < 30 µg/mL after an exposure time of 72 h[41]. In the cytotoxicity assays, the methanol extract of H. fuscum showed remarkable toxicity on both *Artemia salina* larvae (LD_{50} = 3.33 µg/mL) and rhabdomyosarcoma cell (CC_{50} = 8.60 µg/mL) (Table 4), indicating that this extract may possess potent cytotoxic compounds.

Table 4. Cytotoxic acitivty of *H. fuscum*

Extract / compound	BSLA (LD50 µg/mL)	MTT assay (CC50 µg/mL)
H. fuscum	3.33 ± 0.07	8.60 ± 0.02
Cyclophosphamide	0.82 ± 0.02	0.97 ± 0.01

BSLA = brine shrimp lethality assay; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; CC50= 50% cytotoxic concentration

Diseases caused by viruses, especially enteroviruses, are associated with high mortality, as there are no available antiviral agents approved for the management of such infections. The disease burden is also complicated by the lack of good sanitation and hygiene practices in most part of the developing world[42]. Medicinal mushrooms have been reported to display antiviral potentials against several viruses. For instance, triterpenes isolated from *Ganoderma pfeifferi*

displayed potent activity against human immunodeficiency virus type 1 (HIV-1), influenza virus type A and herpes simplex virus type 1[43]. Here, the antiviral activity of the extract of *H. fuscum* was investigated against three serotypes of echoviruses (E7, E13, and E19). With the exception of the E13 strains, *H. fuscum* displayed antiviral activity on the echoviruses under investigation, with an IC50 value of 0.381 and 1.575 µg/mL against the E7 and E19, respectively. In addition, the extract of this mushroom demonstrated good selectivity indexes with better selectivity on E7 (22.57) when compared to E13 (5.46) (Table 5).

Table 5. Antiviral activity of *H. fuscum*

Extract	MNTC (µg/mL)	IC_{50} (µg/mL)			SI	
		E7	E13	E19	E7	E19
H. fuscum	10	0.3811	NA	1.575	22.57	5.46

MNTC: Maximum non-toxic concentration; IC_{50}: 50% inhibition concentration; SI: Selectivity index

Meanwhile, the HPLC analysis of this extract revealed that it contained dihydropenicillic acid as its major constituent, as seen in Figure 1 and 2. Several fungi have been reported to produce dihydropenicillinic acid, and its unsaturated analogue, penicillic acid, and these include *Aspergillus flavus, Aspergillus ochraceus, Aspergillus terreus, Penicillium cyclopium and Penicillium griseofulvum*[44]. A previoius study revealed that dihydropenicillinic acid displayed moderate cytotoxicity against several human cancers including human non-small cell lung cancer, human breast cancer, human CNS cancer and human pancreatic cancer at 10 µg/mL[45].

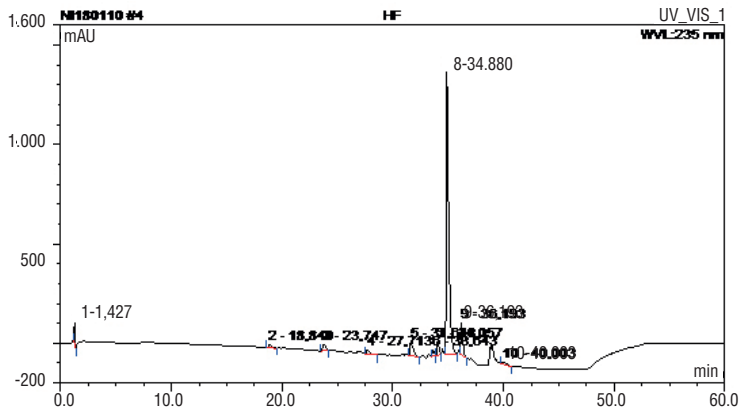

Figure 1 Reverse phase HPLC quantitative chromatogram of the methanol extract of *H. fuscum*

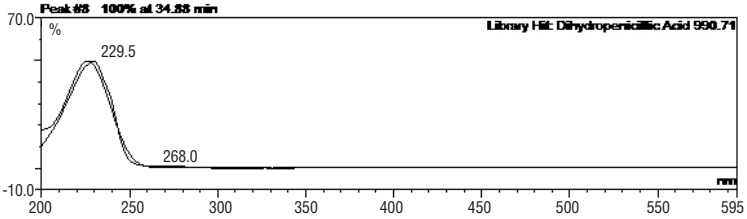

Figure 2 UV spectra assignment of the major peak of the methanol extract of *H. fuscum*

CONCLUSION

This study has demonstrated that extract of the mushroom *Hypoxylum fuscum,* (collected from Nigeria), possesses several pharmacological properties including antiplasmodial, antitrypanosomal, antiviral and cytotoxic properties. To the best of our knowledge, the therapuetic potentials of *H. fuscum* is reported in this work, for the first time. HPLC analysis revealed that dihydropenicillinic acid, which was the major constituent of the extract, may be responsible for the observed biological activities of the mushroom. Considering the diverse biological effects observed in this study, it can be inferred that *Hypoxylum fuscum* may be a source of potential drug lead in the development of effective and affordable chemotherapeutic agents.

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Pharmacological and Pharmaceutical Technological Overview for Seborrheic Dermatitis: A Review About Topical Application and New Approaches

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ABSTRACT

Seborrheic dermatitis (SD) is a skin disease with chronic recurrent erythematous plaques in areas where sebaceous glands are intense. The etiology of SD remains unknown, although many factors such as lipophilic yeasts of the Malassezia genus, hormones, genetic, environmental and general health factors have been shown to be causative. SD is affecting body parts involving rich sebaceous glands such as head and skin. Treatment options include application of anti-inflammatory, antifungal, keratolytic, and sebosupressive agents. The SD causes are briefly mentioned in this review. Then, detailed information was given about the medicines used in SD treatment. The most important element of this article is to be used in SD treatment; new approaches such as new formulations, new active substances and narrow band UV rays have been mentioned. Finally, some examples of the Pharmacist's role in SD treatment in general are given.

Keywords: Seborrheic dermatitis; Formulation; Topical applications

INTRODUCTION

Seborrheic dermatitis (SD) is an amateur skin disease with chronic recurrent erythematous plaques in areas where sebaceous glands are intense.[1] The causes of SD are unknown, but many people with the condition tend to have excessive yeast in their skin in affected areas. Having certain medical conditions can raise the risk for SD. Other conditions that may put someone at risk for SD are: chronic acne, alcoholism or excessive alcohol use, depression, eating disorders, psoriasis and rosacea.[1,2,3]

SD is a chronic inflammatory skin disorder affecting the head, skin, and body. Typically, SD is characterized by dandruff or flaking skin (on the scalp, hair,

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beard, mustache or eyebrows), greasy skin patches (on the scalp, face, nose, eyelids, ears, chest, armpits, groin and under the breasts), inflamed, itchy, reddish skin.[2] In adolescents and adults, SD usually occurs as dandruff, head desquamation and erythema in the nasolabial fold which develops in the newborn period, suggesting that it may be a response to maternal hormone stimulation. SD affects mostly post-pubertal people, but this condition mentioned above also affects a high percentage of infants (around 70 percent) and sometimes children. In fact, cradle cap is one type of seborrheic dermatitis, which affects a baby's scalp. Seborrheic dermatitis can also develop in the diaper area of infants and is sometimes mistaken for diaper rash. In infants, the condition generally resolves by age 3 or 4 months. But children with untreated food allergies can sometimes continue to show symptoms for years. Another time period when seborrheic dermatitis tends to develop is during puberty, when hormone shifts can affect the glands of the skin and oil production. Dermatitis prevalence increases again in adults over the age of 50.[4,5,6,7] Research as additional evidence of hormonal activity has shown that SD emerges in response to androgen stimulation.[2,3] Currently, SDis treated with agents such as antifungal shampoo, topical corticosteroids, topical antifungals, topical calcineurin inhibitors.

In this study, firstly, the causes and properties of SD were briefly examined. The drug groups, topical systems and new aproaches such silver nanoparticles, nanocrystals, new therapeutic agents, narrow-band ultraviolet as used in the treatment are then explained in detail.

Causes of SD

SD is caused by seborrhea, pathologically excessive sebum secretion and infection-inflammation observed afterwards. Lipophilic yeasts, genetic factors, environmental and general health factors contribute to this disorder.[4]

Fungal causes

Although the cause of SD has not yet been fully proven, *Malassezia* that a lipophilic yeast form, may be an inflammatory reaction originating from the spread of yeast. The main species found on the head skin; *M. globosa, M. restricta, M. obtusa, M. sloojjiae, M. sympodialis, M. jurjur, and M. pachydermatis* (the last occurs only on animals). *M. globosa and M. restricta* are thought to be the species most commonly associated with SD, although M. jurjur and other species have also been implicated. Some studies have found high numbers of *Malassezia* yeasts on the scalp of persons with SD, but others have found no difference in the density of these yeasts between the skin of persons with SD and that of persons without it. Differing sampling methods may contribute to these contra-

dictory findings. *Malassezia* exist not only on the skin surface, but also within the layers of the stratum corneum, and a true count would require examining the full thickness of the skin squama. Support for the role of *Malassezia* in SD comes from studies demonstrating that use of various antifungal treatments results in reduction of *Malassezia*, which is accompanied by improvement in symptoms.[1-5]

The toxic substances produced by these yeasts irritate and inflammate the skin. It has been observed that patients with SD have a low resistance to this yeast. However, the proportion of skin affected by the colony may be lower than unaffected skin.[3,4,5]

Thus, a definitive understanding of the pathophysiology of SD awaits further research, but the role of *Malassezia* yeasts as causative or contributing agents appears to be well established.

Other causes

Seborrheic dermatitis commonly occurs among patients with other health conditions that affect their immune systems and raise inflammation. Genetic and environmental factors and other comorbid diseases may lead to the development of SD, which may accelerate the formation of seborrhoeic dermatitis. SD affects only 3 percent of the population. However, this rate can be up to 85% in people with acquired immunodeficiency syndrome (AIDS). The mechanism of HIV (human immunodeficiency virus) infection promoting a typical and explosive seborrhoeic dermatitis and other common inflammatory skin disorders is not fully known; but effective factors such as CD4-positive T-lymphocyte count, *P. ovale* concentration and nutritional factors are still being investigated.[4] Persons with central nervous system disorders (Parkinson's disease, cranial nerve paralysis, large body paralysis) tend to develop SD, in which the disease is more extensive and treatment resistance is observed. In these patients with SD, it has been suggested that the amount of sebum increased due to inactivity resulting in SD. The increased amount of sebaceum supports the development of P. ovale, the cause of SD. Researchers also notes that risk for SD also increases if person taking drugs such as interferon, lithium and psoralen. Even in those who do not have other serious health problems, the risk factors for SD include: stress, trauma, low exposure to sunlight and hormonal changes such as menopause and pregnancy.[2,4,6]

Clinical Signs and Diagnosis of SD

SD is most commonly found in the face and / or head skin and is associated with an unidentified erythematous patch associated with a fine-grained (pitriaziform) scale; one of which is more locating area. Other areas commonly affected

by SD; forehead parts of the forehead, anterior hairline area, glabella areas of the forehead, nasal folds, melolabial folds, ears (external ducts, anterior auricular region, retroauricular region), sternum-centered chest area and the genital area.[4,6,7] As mentioned earlier, SD usually has symmetrical distribution in these areas.[4]

One of the features of SD is the blemish on the bran with a thin, powder-white scale. Many patients complain of the scalp itching with dandruff, and because they think that the scale arises from dry skin, they decrease the frequency of shampooing, which allows further scale accumulation. Inflammation then occurs, and their symptoms worsen.[4,5,6]

More severe SD is characterized by erythematous plaques frequently associated with powdery or greasy scale in the scalp, behind the ears and elsewhere in the distribution described above. Besides an itchy scalp, patients may complain of a burning sensation in facial areas affected by seborrhea. Seborrhea frequently becomes apparent when men grow mustaches or beards and disappears when the facial hair is removed. If left untreated, the scale may become thick, yellow and greasy and, occasionally, secondary bacterial infection may occur.[4]

SD is more common in men than in women, probably because sebaceous gland activity is under androgen control. Seborrhea usually first appears in persons in their teens and twenties and generally follows a waxing/waning course throughout adulthood.[4]

The differential diagnosis of SD should include psoriasis, rosacea, Demodex dermatitis, atopic eczema, pityriasis versicolor, contact dermatitis, and tinea infections. SD may also resemble Langerhans cell histiocytosis or secondary syphilis. The diagnosis is usually clinical, but candidiasis, tinea infection, and Demodex dermatitis may be ruled out with a negative potassium hydroxide test. It should be kept in mind that SD may be accompanied by other dermatological disorders.[4]

Care should be taken to differentiate SD from psoriasis vulgaris. Early SD has a spongiform appearance that distinguishes it from psoriasis, but in later stages these conditions are more difficult to tell apart. Some patients present with sebopsoriasis, which includes features of both disease states.2 Lesions on the elbows or knees and nail pitting suggest psoriasis, which may spare the face.[4]

Finally; the most common signs and symptoms of SD are:[1, 4, 8, 9]

• Scaly patches and dandruff forming on the scalp. Dandruff is usually “stubborn” and doesn’t respond well even to over-the-counter products like dandruff shampoos or oily treatments. Sometimes, rash-like lesions form on the scalp

that can range from mildly itchy to brownish crusts and cause the skin to stick to the hair.

• Red skin that's sensitive and very dry. Irritated SDcan cause very dry skin to become yellow or pink in certain areas.

• Extra dryness and flaking in areas of the skin that fold or that are usually oily, including the "T-zone" of the face (the area covering the nose, chin and forehead), upper chest and back.

• Skin peeling called erythema, which sometimes appears as macules (a discolored patch of skin) or plaques with dry white or moist oily scales. Even when skin peels, papules or pustules are not very common.

• It's possible for secondary bacterial infections to occur if bacteria proliferate inside dry patches or openings of the skin.

Treatment of SD

Hygiene plays a key role in controlling SD. Frequent cleaning with soap will remove the fat in the affected areas and help to heal SD. Patients should be informed that hygiene conditions are very important for a good life. The so-called 'open-air reaction' observed especially during the summer will increase seborrhea and precautions must be taken to prevent sun damage.[3, 4, 7]

There are anti-inflammatory agents (topical steroids) and antifungal agents (selenium sulfide, zinc pyrite, azole agents, sodium sulfacetamide, topical terbinafine etc.) that reduce lipophilic yeast colonization as a pharmacological treatment option for SD. In severe disease states, keratolytic agents such as salicylic acid and tar preparations may be used to remove intense scaling followed by treatment with topical steroids.[4, 8, 9]

Sebosupressive agents such as isotretinoin can be used as a last resort in the problem of resistant SD to reduce sebaceous gland activity.[3]

Drugs used in the treatment of SD are summarized in next section. This information was supported by RxMediaPharma® and the literature. RxMediaPharma® is a comprehensive drug information resource. The goal is to support doctors, pharmacists and other health professionals in their current knowledge, to inform them about new developments and to ensure that information is delivered quickly.[59]

Drugs for SD

Beclamethazone dipropionate: It is used topically in the treatment of certain skin diseases with severe inflammation, such as eczema, which does not

respond to weaker corticosteroids. There are pomade, cream and lotion forms.[10]

Betamethasone: It is anti-inflammatory and antipruritic medicine. It is used in the treatment of dermatoses that respond to topical corticosteroid therapy.[11]

Betamethasone valerate: It is used against the inflammation seen in dermatoses that respond to corticosteroid treatment. It has anti-inflammatory and antipruritic effect.[12]

Cetrimide: This drug is a quaternary ammonium derivative and antiseptic. Shampoos containing 1 to 3% of cetirimide are used against perspiration in case of SD.[13]

Clobetazol propionate: This drug is an effective topically applied synthetic fluorocorticosteroid. It is used in the treatment of inflammatory and pulmonary symptoms caused by moderate to severe dermatoses and psoriasis that respond to corticosteroids.[14]

***Clobetazon butyrate*:** This drug is an effective topically applied synthetic fluorocorticosteroid. It is an anti-inflammatory corticosteroid that is used in inflammatory conditions not related to infection, especially in the treatment of eczematous disorders. It is used in all kinds of eczema and dermatitis cases. More powerful corticosteroid drugs are used for treatment of idiopathic remedies. There are 0.05% creams and ointments.[15]

Dexamethasone, Dexamethasone sodium phosphate: They have anti-inflammatory effect. When given in pharmacological doses, they prevent inflammation and immune responses.[16, 17]

Dezonide: It has anti-inflammatory effect. Dezonide (prednasinolone) is a prednisolone derivative corticosteroid used topically to treat various skin diseases. Acute and chronic eczema responding to topical corticosteroid therapy; neurodermatitis; contact, chronic and SD; dermatitis; chronic lichen simplex; hypertrophic lichen planus; anogenital and senile pruritus; psoriasis and other similar cases.[18]

Diflucortolone valerate: It is a powerful corticosteroid used topically for the treatment of various skin diseases. It is used in the treatment of certain skin diseases with severe and resistant inflammation, such as stubborn eczema, which does not respond to less powerful corticosteroids. It is also indicated in cases such as dermatomycosis due to yeast and yeast-like fungi causing SD, tinea and candidiasis.[19, 20]

Flucortolone hexanoate, Flucortolone pivalate: These drugs have anti-inflammatory effect. They are used in contact dermatitis, SD, various eczema

types, neurodermatitis, psoriasis, lichen ruber planus, atopic dermatitis, burns and insect infestations. It is used in cream and pomade forms.[21, 22]

Flumethazone pivalate: This drug has anti-Inflammatory effect. It is used to treat various inflammatory skin diseases such as various eczema that respond to topical corticosteroid treatment. Cream, pomade, lotion forms are available.[23]

Flucinolone acetonide: This drug has anti-Inflammatory effect. It is a topically applied chlorinated corticosteroid. Corticosteroids are used in the treatment of susceptible dermatoses and psoriasis. There are forms of cream, pomade, topical solution, shampoo and topical oily solution.[24]

Flusinonid: When this drug administered topically, the inflammation caused by acute and chronic dermatoses is abolished by anti-inflammatory, antipruritic and vasoconstrictor effects specific to topical corticosteroids. It is used in the symptomatic treatment of skin diseases characterized by inflammation and / or pruritis, such as SD, atopic dermatitis and psoriasis which respond to corticosteroid treatment in the form of cream, gel, solution and lotion.[25]

Fluticasone propionate: This drug has anti-inflammatory, antipruritic, vasoconstrictor effects. It is a synthetic corticosteroid with medium strength. It is used against inflammatory and pruritic symptoms of dermatosis and psoriasis which respond to topical corticosteroids. The antiflammatory activity of topical fluticasone is 13 times that of triamnisolone acetonide, 9 times that of flucinolone acetonide, 3 times that of betamethasone valerate and 2 times that of beclomethasone propionate.[26]

Halometasone: It is a potent corticosteroid administered topically. This drug has anti-inflammatory, antiallergic, vasoconstrictor and antiproliferative effects. It is usually used against inflammatory skin diseases of various types and origins. These include inflammatory skin diseases such as non-infected SD, contact dermatitis, atopic dermatitis, localized neurodermatitis (lichen simplex cronicus), numeral eczema and psoriasis vulgaris, which respond to corticosteroid therapy. For this purpose, creams and ointments containing 0.05% halomatazon are used.[27]

Halsinoid: The topically used steroid is a highly fluorescent synthetic corticosteroid. Corticosteroid-sensitive dermatosis is used in the treatment of inflammatory and pruritic symptoms of psoriasis. Anti-inflammatory, antipruritic and vasoconstrictor effect. inflammation and pruritus associated with acute and chronic dermatoses.[28]

Hydrocortisone, Hydrocortisone acetate: It is a steroid hormone that secretes adrenal cortex. The preparations are in the form of natural hormones

as well as their derivatives. Like topical corticosteroids, hydrocortisone has anti-inflammatory, antipruritic and vasoconstrictor effects. Cream, cream, lotion-like preparations are available.[29, 30]

Hydrocortisone butyrate: It has anti-inflammatory, antiproliferative, vasoconstrictor, antiallergic and antipruritic effect. There are various pharmaceutical forms which are prepared according to the type and degree of the skin disease and which have the effect of helping the skin.[31]

Itraconazole: It is an antifungal medication that is similar to ketoconazole in the oral route. The effectiveness of the treatment of dermatophyte infections in the skin that do not respond to topical treatment is being investigated.[32]

Juniper tar (cade oil): This antiseptic drug is used as an ointment directly on the skin. It is also used externally for the treatment of eczema and psoriasis. This drug is used to treat seborrhea, eczema, psoriasis, dandruff, scalp and pruritus. This drug enters the combination of shampoos prepared for this therapeutic purpose.[33]

Ketoconazole: It is an antifungal drug included in the imidazole group. Ketoconazole is the only drug that is capable of inhibiting the synthesis of adrenal steroids at higher doses than the doses required for antifungal activity. Oral tablet, topical cream (2%) and shampoo (2%) are available. There is also a 1% shampoo for use against the head.[34]

Methylprednisolone, Methylprednisolone acetate: These drugs are an anti-inflammatory and immunosuppressive agent. Pharmacology is the natural hormone that prevents or suppresses inflammation and immune responses when given in doses.[35]

***Mometasone furoate*:** This drug is a moderate synthetic corticosteroid with anti-inflammatory, antipruritic and vasoconstrictor properties. It is used topically against dermatoses that respond to corticosteroids and inflammatory and pruritic symptoms due to psoriasis.[36]

Prithion zinc: This medicine is used in the treatment of simple dandruff, dry and oily type of SD. For this purpose 2% concentration is applied. It goes into the combination of the shampoos used against the head. The mechanism of action has not been fully elucidated. According to one opinion, it has bacteriostatic and fungicidal action. According to another opinion, it shows cytostatic effect on decrease of turnover rate in epidermal cells.[37]

Prednicarbat: This drug is a synthetic topical corticosteroid with no halogen and is moderately effective. This drug is used to treat the inflammatory and

pruritic symptoms of dermatosis and psoriasis that respond to corticosteroid therapy.[38]

Prednisolone, Prednisolone acetate: These drugs are anti-inflammatory and immunosuppressive synthetic glucocorticoid.[39, 40]

Salicylic acid: It's a kerolytic drug. It is used in the treatment of diseases such as dandruff, SD, ichthyosis, psoriasis and acne with hyperkeratotic and deep scaling. Solutions and pomades are used for this purpose. Salicylic acid reduces desquamation and is slightly fungicidal.[41, 42]

Selenium sulfide: It's an antifungal and antiseborrheic agent. Shampoos are used in the treatment of dandruff, SD and tinea versicolor. Antiseborrheic effect is due to cytostatic effect on cells in epidermis and follicular epithelium. Used topically. 1% shampoos for dandruff treatment, 2. 5% shampoo or lotions for SD treatment.[7, 9]

Topical pharmaceutical forms for the treatment of SD

With the exception of severe SD treatment, it can be treated with pharmaceutical forms such as cream, lotion, ointment, pomade, shampoo, solution, suspension in the scalp, face, beard zone and body in the treatment of SD. These pharmaceutical forms and examples will be examined separately in the next section.

Cream

Topical drug delivery can be defined as the administration of a drug containing the formulation to the skin. It can be used primarily to treat cutaneous manifestations of a universal disease such as cutaneous diseases such as acne or psoriasis and for many purposes. The main purpose of this transmission is to limit the pharmacological or other effect of the drug to the skin surface or deeper skin layers.[43, 44, 45] Creams, which are a semi-solid emulsion formula, are applied to skin or mucous membranes. Oil-in-water (w/o) emulsion type creams are less oily and well spread than ointments. Water-in-oil (o/w) emulsion type creams are easily applied to the skin and are easily removed with water.[44] Antifungal, corticosteroid or combination cream formulations used in the treatment of SD are presented in Table 1.

Table 1. Topical cream preparations currently used in Turkey for SD.

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Beklazon, cream, 30 g tube/box	Beclamethazone dipropionate 0.025 %
Betnovate, cream 30 g tube/box	Betamethasone valerate 0.1 %
Betnovate-C, cream, 30 g tube/box	Betamethasone valerate 0.1 % Clioquinol %3
Codermo, cream, 30 g tube/box	Mometasone furoate 0.1 %
Dermatop, cream, 30 g tube/box	Prednikarbat 0.25 %
Dermovate, cream, 50 g tube/box	Clobetazol propionate 0.05 %
Diprolene, cream, 15 g tube/box	Betamethasone dipropionate 0.05 %
Eumovate, cream, 50 g tube/box	Clobetazon butyrate 0.05 %
Fucicort, cream, 30 g tube/box	Fusidic acid 2 % Betamethasone valerate 0.1%
Hexacorton, cream, 30 g tube/box	Prednisolone acetate 0.5 %
Hipokort, cream, 30 g tube/box	Hydrocortisone acetate 0.5 %
Impetex, cream,10 g tube/box	Diflucortolone valerate % 0.1, Chlorquinaldol %1
Ketoderm, cream, 30 g tube/box	Ketoconazole 2 %
Locacortene-Vioforme, cream, 15 g tube/box-30 g tube/box	Flumetazone pivalate 0.02 % Clioquinol 3%
Locoid, cream, 30 g tube/box	Hydrocortisone 17-Butyrate 0.1 %
Locoid, lipocream, 30 g, tube/box	Hydrocortisone 17-Butyrate 0.1 %
M-Furo, cream, 30 g tube/box	Mometasone furoate 0.1 %
Nerisona C, cream, 15 g tube/box	Diflukortolone valerate 0.1 %, Chlorquinaldol %1
Nizoral, cream, 30 g tube/box	Ketoconazole 2 %
Prednol, cream, 30 g tube/box	Prednasinolon 0.125 %
Prednol-A, cream, 30 g tube/box	Prednasinolon 0.125 %, Iodochlorhydroscine 3 %
Sicorten, cream, 30g tube/box	Halomethasone 0.05 %
Tetemex, cream, 10 g tube/box	Diflukortolone valerate 0.1%
Ultralan, cream, 20 g tube/box	Flucortolon caproate 0.25 % Fluconthalon 0.25%
Ureacort, cream, 30 g tube/box	Urea 5% Hydrocortisone acetate 0.05 %
Hydrocortisone acetate 0.05 %	Urea 10 % Hydrocortisone acetate 0.05 %

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Lotion

Lotion is a low to moderately viscous topical preparation for application to unbroken skin. Lotions are applied to the skin with bare hands, with a clean cloth, cotton or gauze, and provide cooling effects on the skin by solvent evaporation.[44] Lotions are liquid preparations in which a finely divided insoluble drug is suspended or dispersed. They are easily applied to the hair planting areas and spread easily.[46] Corticosteroid or combination lotion formulations used in the treatment of SD are presented in Table 2.

Table 2. Topical lotion preparations currently used in Turkey for SD.

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Beklazon, lotion, 50 mL bottle/box	Beclamethazone dipropionate 0.025 %
Betasalic, lotion, 50 mL bottle/box	Salicylic acid 2 % Beclamethazone dipropionate 0.05%
Betnovate, lotion 20 mL bottle/box	Betamethasone valerate 0.1 %
Betnovate, hair lotion, 30 mL bottle/box	Betamethasone valerate 0.1 %
Codermo, lotion, 30 mL bottle/box	Mometasone furoate 0.1 %
Dermovat, Hair lotion, 25 mL bottle/box	Clobetazol propionate 0.05 %
Elocon, lotion, 30 mL bottle/box	Mometasone furoate 0.1 %
M-Furo, lotion, 30 mL bottle/box	Mometasone furoate 0.1 %
Psoderm, hair lotion, 25 mL bottle/box	Clobetazole propionate 0.05 %

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Ointment

Ointments are biphasic devices believed to be occlusive, which prevents transcutaneous water loss and water trapping on the skin surface.[46] It is a greasy semisolid preparation of dissolved or dispersed drug. Ointment bases influence topical drug bioavailability due to their occlusive properties of the stratum corneum, which enhances the flux of drug across the skin and they affect drug dissolution and drug partitioning within or from the ointment to the skin, respectively[44]. Antifungal, corticosteroid or combination ointment formulations used in the treatment of SD are presented in Table 3.

Table 3. Topical oinment preparations currently used in Turkey for SD

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Betasalic, ointment, 30g tube/box	Salicylic acid 3 % Beclamethazone dipropionate 0.05%
Betnovate, ointment 30 g tube/box	Betamethasone valerate 0.1 %
Betnovate-C, ointment, 30 g tube/box	Betamethasone valerate 0.1 % Clioquinol %3
Codermo, ointment, 30 g tube/box	Mometasone furoate 0.1 %
Cutivate, ointment, 30 g tube/box	Fluticasone propionate 0.005 %
Dermatop, ointment, 30 g tube/box	Prednikarbat 0.25 %
Dermovate, ointment, 50 g tube/box	Clobetazol propionate 0.05 %
Eumovate, ointment, 50 g tube/box	Clobetazon butyrate 0.05 %
Heksa, ointment, 15 g tube/box	Polymyxin B sulfate 0.1 % Oxytetracycline hydrochloride 3 %
Ketoral, ointment, 40 g tube/box	Ketoconazole 2 %
Locasalene, ointment, 30 g tube/box	Flumetazone pivalate 0.02 % Salicylic acid 3%
Locoid ointment 30 g tube/box	Hydrocortisone 17-Butyrate 0.1 %
M-Furo, ointment, 30 g tube/box	Mometasone furoate 0.1 %
Polimisin, ointment, 14 g tube/box	Polymyxin B sulfate 0.1 % Oxytetracycline hydrochloride 3 %
Psovate, ointment, 50 g tube/box	Clobetazole propionate 0.05 %
Tetemex, ointment, 10 g tube/box	Diflukortolone valerate 0.1%
Terramycin, ointment, 14.2 g tube/ointment	Polymyxin B sulfate 0.1 % Oxytetracycline hydrochloride 3 %

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Pomade

Pomades are externally used semi-solid drug delivery systems prepared to be applied on the skin. There are O/W emulsion types and they contain high oil (70%). Therapeutically effective ointments have mildly softening and protective properties and additionally contain active ingredients with therapeutic effect. They have deep penetrating abilities. They do not clench the skin pores.[47] Corticosteroid or combination ointment formulations used in the treatment of SD are presented in Table 4.

Table 4. Topical pomade preparations currently used in Turkey for SD.

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Beklazon, pomade, 30 g tube/box	Beclamethazone dipropionate 0.025 %
Diprolene, pomade, 15 g tube/box	Betamethasone dipropionate 0.05 %
Elocon, pomade, 30 g tube/box	Mometasone furoate 0.1 %
Hipokort, pomade, 30 g tube/box	Hydrocortisone acetate 0.5 %
Kenacort-A, pomade, 20 g tube/box	Triamnisolone acetonide 0.1 %
Prednol, pomade, 30 g tube/box	Prednasinolon 0.125 %
Prednol-A, pomade, 30g tube/box	Prednasinolon 0.125 % Iodochlorhydroscine 3 %
Seroderm, pomade, 30 g tube/box	Betamethasone valerate 0.1 %
Thiocilline, pomade, 30 g tube/box	Bacitrasyn IU/g Neomycin sulphate 0.5 %
Ultralan, pomade, 20 g tube/box	Flucortolon caproate 0.25 % Fluconthолon 0.25%

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Shampoo

When shampoos are first discovered, shampoos are defined as effective cleansing agents for hair and scalp, but today this is not just what is expected from shampoo today. Although the functions of shampoos have become complicated in recent years, their main purpose is to clean the hair by removing sebum and foreign residues from the surface of the hair.[48, 49, 50] Shampoos are also effective in the following cranial conditions; repair of alkaline neuralization ability, dryness, seborrhea, head skin infections *(Malassezia spp., Propionibacterium spp.)*, the enzyme activation state on the head skin, arrhythmia of the head skin.[48] Antifungal or combination shampoo formulations used in the treatment of SD are presented in Table 5.

Table 5. Topical shampoo preparations currently used in Turkey for SD.[59]

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Fungoral, shampoo, 100 mL bottle/box	Ketoconazole 2 %
Ketoral, shampoo, 100 mL bottle/box	Ketoconazole 2 %
Konazol, shampoo, 100mL bottle/box	Ketoconazole 2 %
Nizoral, shampoo, 60 mL bottle/box	Ketoconazole 2 %
Selsun blue, shampoo, 200 mL bottle/box	Selenium sulfide 1 %
T/GEL, shampoo 130 mL/ 251 mL bottle/box	Coal tar 2%

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Solution

Solvents are a homogeneous mixture prepared by mixing two or more substances, that is, a mixture dissolved or dispersed all around the same.[51] Solution formulations used in the treatment of SD are presented in Table 6.

Table 6. Topical solution preparations currently used in Turkey for SD.

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Dermatop, solution, 20 mL bottle/box	Prednikarbat 0.25 %
Ultralan, solution, 20 mL bottle/box	Flucortolon pivalate 0.5 % Salicylic acid %1

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Suspension

Suspensions are heterogeneous systems containing two phases. The external phase, also referred to as the continuous phase or dispersion medium, is usually a liquid or semi-solid and the internal or dispersed phase is composed of particulate matter that is substantially insoluble in the external phase. Most drug suspensions consist of an aqueous dispersion medium, but in some cases organic or oily fluids are also used.[52] A suspension formulation used in the treatment of SD are presented in Table 7.

Table 7. Topical suspension preparation currently used in Turkey for SD.[59]

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Zetion, suspension, 100 mL bottle/box	Zinc pyrite 2%

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

Gels

The gel is a transparent or translucent semi-solid preparation in which one or more active substances are transported in suitable hydrophilic or hydrophobic bases. The gel may be clear or opaque, polar hydroalcoholic or nonpolar. Gels are either prepared by a special procedure required by a fusion process or by gelling agents, humectants and preservatives.[53] A suspension formulation used in the treatment of SD are presented in Table 8.

Table 8. Topical gel preparation currently used in Turkey for SD.[59]

The name of the preparation, pharmaceutical form, amount	Active ingredients and ratio
Pers-mant, gel, 30 g bottle/box	Miconazole nitrate 2 % Aluminum hydroxychloride 19 %

* Information was obtained from the RxMediaPharma® interactive drug information resource.[59]

New approaches

Dandruff is a common haired skin problem of human beings, and most people suffer from this at some point in their lives. To prevent dandruff, pre-dandruff shampoos have become popular in recent years. However, in many cases, Malassezia furfur, which produces dandruff and is a cause of SD, develops resistance to widely used anti-dandruff drugs. As a result, a new class of pre-dandruff shampoos needs to be developed for dual purposes. In a study, it was mainly focused on a green nanoparticle-based green formulation of a pre-dandruff shampoo. *In situ* capped silver nanoparticles (Ag NPs) were prepared by green pathways using *Acacia and Acacia + Aegle marmelos* leaf extracts (LE) at sizes 40 and 13 nm, respectively. Anti-fungal activity tests using a well diffusion technique showed that the 13 nm particles had better inhibitory activity compared to 40 nm particles, while the TTC (2,3,5-triphenyl-tetrazolium chloride) analysis method showed a minimum or threshold concentration of 0.054 mM for both particle sizes used. The time-kill assay shows some synergism in the net antifungal effect of Acacia + LEs in aqueous media against M. furfur and in 13 nm Ag NPs. However, the promising anti-Malassezia activity of 40 nm Ag NPs in Acacia medium and their superior suspension stability against microbial contamination means that they have potency as an active and simple antifungal shampoo formulation.[54]

In another study about silver nanoparticles, a detailed analysis of the treatment of hair-dermatitis infection using silver nanomaterials (AgNMs) is summarized and focuses on biocide activity due to manipulation of size, shape and structure. Monodisperse silver spherical nanoparticles (NPs) and nanorods (NRs) were synthesized chemically and characterized using analytical / spectroscopic techniques. Ag NMs showed more biocidal tendency and showed more inhibition zone compared to drugs in SD treatments, itraconazole and ketoconazole. Obtained 20 nm and 50 nm spherical NPs and 50 nm NRs showed concentration, size, and shape-dependent antifungal activity; 20 nm spherical NPs exhibited excellent potency. The minimum inhibitory concentration for 20 nM was the lowest at 0.2 mg/mL compared to 0.3 mg / mL for NRs. The NMs with a 50 nm rod showed negligible redness, whereas the primary irritation index was 0.33 and 0.16 for 20 nm and 50 nm spherical NPs, respectively. An in vivo model for *M. furfur* infection was produced by passing fungi subcutaneously into rat skin. Again, 20 nm particles showed the best normalization of the skin after 10 days at regular dosing compared to larger and stick-shaped particles. The statistical clinical score was highest for Ag nanorods, followed by the animal treated with 50 nM Ag NPs. 20 nm spherical particles exhibited the lowest score (0) as compared to the antifungal drugs as well as others. Biochemical analysis with anti-

oxidant enzymatic activity control, tissue repair and enzymatic normalization and protein NP concentrations were shown.[55]

In another study about silver nanoparticles, nanoparticles (AgNP) were synthesized using *Coriandrum sativum* leaf extract. The physico-chemical properties of AgNPs have been analyzed by various analytical and spectroscopic methods. In addition, the in vitro anti-acne, anti-dandruff and anti-breast cancer efficacy of synthesized AgNPs were respectively assessed against cell lineage of Propionibacterium acnes MTCC 1951, Malassezia furfur MTCC 1374 and human breast adenocarcinoma (MCF-7), respectively. The flavonoids contained in the plant extract were responsible for AgNP synthesis. The size of the green synthesized nanoparticles was found to be ~ 37 nm. The BET (Brunauer-Emmett-Teller) analysis revealed that the surface area of synthesized AgNPs was 33.72 m^2 g^{-1}. It was found that the minimum inhibitory concentration (MIC) of AgNPs for M. furfur, which is the causative agent of P. acnes and dandruff, is 3.1 and 25 μg mL-1, respectively. The half-maximal inhibitor concentration (IC_{50}) of AgNPs for MCF-7 cells was calculated to be 30.5 μg mL-1and a complex inhibition at 100 μg mL^{-1} concentration was observed. Finally, the results can be interpreted as evidence that green-synthesized AgNPs using C. sativum have great potential in biomedical applications such as anti-acne, anti-dandruff, anti-SD and anti-breast cancer therapy.[56]

Nanocrystals are nanoparticles composed of 100% drug without any matrix material, typically with a size range between 200 and 500 nm.57 In a study of nanocrystalline nanoparticles, ketoconazole nanoparticles were synthesized from ketoconazole powder in the market. Sonication has been preferred as the physical method used in the manufacture of ketoconazole nanoparticles. Particles analyzed by different analytical and spectroscopic methods reveal the formation of multi-parted ketoconazole nanoparticles of 51nm particle size. In the antifungal activity study, it has been shown that synthesized ketoconazole nanoparticles exhibit significant activity against SD causes Malassezia furfur when compared to ketoconazole powder in the market. Furthermore, nanogel was prepared using ketoconazole nanoparticles with significant antimicrobial and antimalassezial activity. After systematic testing, the gel containing ketoconazole nanoparticles can be used as an anti-dandruff gel.[58]

Another of the new therapeutic approaches is tacrolimus and pimecrolimus from the nonsteroidal macrolactamine immunomodulator group. These active ingredients have been tested on SD and found to be effective. These active ingredients act by inhibiting calcineurin. It is thought that these drugs may be useful in the treatment of SD because of the anti-inflammatory effects that they have.[60, 61, 62]

In vitro studies have suggested that tacrolimus may exhibit antifungal activity against *P. ovale* as another potential mechanism of action.[25, 60] These agents have a number of known side effects, including stinging, cough, fever, skin atrophy, burning, heading, upper respiratory infection and flu-like symptoms (among others). Not much is known about the long-term effects of calcineurin inhibitors. Those medications come with warnings that isolated cases of skin cancer and lymphoma have occurred. Never use these medications long-term under any circumstances.[60]

Another new approach to SD therapy is lithium compounds. Lithium succinate pomade with 8% lithium succinate and 0.05% zinc sulphate is available in some countries.[63] Rapid improvement was achieved with lithium succinate pomade that administered twice a day in patients with AIDS and facial SD together.[64] In another study, lithium succinate pomade has been shown to be effective in HIV negative SD cases.[65] Lithium succinate pomade has a potent antiviral effect and was first developed as a possible treatment for oral and genital herpes. Treatment effect on SD was found by chance. This drug proved to be sufficient alone to control SD in all areas except the scalp[64]. In vitro studies have shown that lithium salts inhibit yeast proliferation. It is interpreted that this effect may be mainly related to the inhibition of free fatty acid production. Lithium salts also have an antiinflammatory effect by inhibiting arachidonic acid production.[66] While some authors suggest that lithium succinate is effective against malassezia species in vitro, others have reported that they have anti-inflammatory effects rather than a specific antifungal agent.[63,66]

In another study aimed to investigate the efficacy of narrow-band ultraviolet (UV) B (TL-01) phototherapy as an alternative treatment for SD. Eighteen patients with severe disease were enrolled in an open prospective study. Treatment was given three times weekly until complete clearing or to a maximum of 8 weeks. A clinical score assessing erythema, scaling, infiltration and pruritus was performed at baseline and every 2 weeks thereafter. Additionally, the patients were asked to rate the intensity of pruritus on a visual analogue scale. After completion of the study the patients were followed up to determine the median time interval until recurrence. All patients responded favourably to treatment, with six showing complete clearance and 12 marked improvement. The median clinical score decreased from 7.5 (range 4±8) at baseline to 0.5 (range 0±3) after 8 weeks of treatment. The median pruritus score decreased from 4.5 (range 0±8) at baseline to 0 (range 0±3) at week 8. Relapses occurred in all patients after a median of 21 days (range 12±40). No side-effects of treatment were observed except occasional episodes of a moderate erythemal response. As a conclusion

this study, results demonstrate that narrow-band UVB is an effective alternative tool for the management of severe SD and should be considered for patients unresponsive to conventional treatments.[67] The mode of action of narrow-band UVB in SD remains speculative yet presumably is related to its modulatory effect on inflammatory and immunological processes in the skin. In addition, a direct effect of UV irradiation on *P. ovale* leading to ultrastructural changes and growth inhibition has been reported.[68, 69]

In one study, '1-24 dihydroxycholecalciferol' creams were applied twice a day for 4 weeks in patients with SD on their face and scalp. At the end of treatment all the lesions were seen to be regressed. No recurrence was observed at 2 months follow-up.[70]

Role of Pharmacist

Since SD is a condition that is visible, patients normally feel humiliated and may see themselves as distorted. Patients can profit significantly from the contribution of a pharmacist. The pharmacist ought to acquire a prescription history to ascertain the seriousness of the patient's manifestations, at that point offer guiding about the most proper over-the-counter regimen. Pharmacist ought to talk about the objectives of treatment, reasonable desires, length of treatment, suitable utilization of items, the significance of clinging to the regimen, and any conceivable unfriendly impacts.[9]

To diminish the danger of medication associations, the pharmacist ought to keep up refreshed prescription profiles for every patient, including the utilization of home grown items, nonprescription medications, and regular supplements. Also, the pharmacist should screen for attendant medications with a limited remedial file or medications that may collaborate with different medicines. The scope of treatments for SD can be overpowering to patients, however a pharmacist can help locate the fitting therapy or prompt them when discussion with a dermatologist is justified.

Initially it is important that patients get accurate information from pharmacists in skin diseases. In conclusion, although there are some identifiable opportunities to provide information to pharmacologic dermatology patients, it is important that they have adequate training.

Pharmacists must be fully trained and integrated to provide a comprehensive pharmaceutical care package for dermatology patients to require time.

"The task of the pharmacist is to give self-referral advice on dermatological conditions to people who have not seen a doctor, based on current information on

over-the-counter (OTC) products. For example, health professionals recommend that people with eczema treat themselves by using a weak corticosteroid and emollient, and then refer people to a doctor if the eczema is severe or the cure fails.

The role of the pharmacist in therapeutic support seems unclear and probably not used enough. It has been suggested that the pharmacist is often the first and last provider they have encountered in the process up to the sum of the pills prescribed by the patient's skin treatment. In addition to the doctor and nurse, pharmacists may be more active following treatment in cooperation with other health professional. More drug treatment problems lead to the identification and resolution of a drug when it is examined by a pharmacist, which is only compared with patients who have regular contacts with their physician. This can reduce the direct cost of medicines and other health care use. Practical problems related to the application of topical preparations, lack of motivation and compliance, potential side effects and inadequate efficacy are problems that can be detected by the pharmacist.

The pharmacist's recommendations for infantile SD and adult SD cases should be different. In infants, SD is the primary treatment, to keep the skin dry and to relieve inflammation. Pharmacist should be recommended to families of infants suffered SD, such as diapers should not be used in hot weather, soap and puddle, or because they irritate eyes, should be avoided from tar preparations. Careful and moderate treatment regimens are recommended because the disease has a long course in adults. Pharmacists should be advised of the use of antiinflamatuaries, antimicrobial or antifungal agents, if necessary, and personal hygiene in adults exposed to SD.[64]

Pharmacist has a great role in directing patients about antagonistic impacts, treatment results, consistence, and the fitting utilization of their recommended treatments of SD.

CONCLUSION

SD is a continuous disease spectrum that affects the seborrhoeic regions of the body. Various internal and environmental factors such as malassezia yeast, host epidermal conditions, sebaceous secretion, immune response and interactions between these factors may contribute to pathogenesis. Effective management of SD requires symptomatic relief, such as pruritus and overall hair and skin health, to help clear symptoms with antifungal and anti-inflammatory therapy, to help maintain remission. Studies in humans and animal models to investigate genetic and biochemical pathways will help identify new targets for the devel-

opment of more effective treatment with fewer side effects and better manage these conditions. As it is understood from this review article; many active agents and traditional pharmaceutical formulations are still used in the treatment of SD. Besides, new pharmaceutical formulations and new agents to be used in SD treatment are being developed and their efficacy proved even if the market has not yet entered the market.

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Flurbiprofen Loaded Gel Based Topical Delivery System: Formulation and *In Vitro* Characterization with New Developed UPLC Method

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ABSTRACT

Objective: The purpose of this study was to formulate flurbiprofen (FLB) loaded methylcellulose (MC), hydroxypropyl methylcellulose (HPMC) and Carbopol®940 (C-940) based gel formulations with the help of dispersion method for topical application. Additionally, in this study also a new ultra performance liquid chromatography method was developed for the determination of FLB, which was not previously entered into the literature.

Method: FLB loaded gel formulations with the help of dispersion method for topical application and to characterize the formulations according to physical appearance, pH, rheology, drug content, dissolution study and release kinetic study with the DDSolver software program. The UPLC method developed was validated for linearity, specificity, precision, sensitivity, accuracy, range and robustness.

Results: Linearity was determined to be at a concentration range of 5-50 $\mu g.mL^{-1}$. The method developed for FLB was decided to be precise due to RSD values of <2%. Recovery of the method was satisfactory owing to <2%RSD value. The drug content was found to be in the range of 98.14-99.02% indicating the uniformity of the high drug content. At the 6th hour in dissolution study, the FLB release from gels prepared with MC, HPMC, C-940 reached 99.7%,99.5% and 87.60%, respectively. In the release kinetic tests with DDSolver, the release of gels prepared with MC and HPMC showed conformity with the weibull model, whereas the gel formulation prepared with C-940 showed a zero-order kinetics.

Conclusion: According to the results, all gel formulations prepared have longer release times than the release of pure FLB.

Keywords: Flurbiprofen, UPLC, Topical Gel, DDSolver, Release Kinetics

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most popular drugs in the world because of their efficacy in reducing pain and inflammatory reactions. NSAIDs have been documented worldwide for use in many clinical situations such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout, dysmenorrhea, toothache and headache.[1] The main pharmacological effect of these group of drugs is the inhibition of the pro-inflammatory enzyme cyclooxygenase (COX). NSAIDs are divided into two groups. The first group is traditional non-selective NSAIDs that specifically inhibit both COX-1 and COX-2. The other group is selective COX-2 inhibitors.[2] Flurbiprofen (FLB) belongs to the first group of NSAIDs with a molecular weight of 244.3 g/mol. FLB is commercially available as racemate blend of (+) S and (-) R-enantiomers. FLB is poorly water soluble but soluble in DMS and ethanol.[3] Different high performance liquid chromatography (HPLC) methods have been introduced into the literature for the determination of FLB quantities in various biological fluids and pharmaceutical dosage forms.[4,5,6,7,8] However, the ultra performance liquid chromatography (UPLC) method is not yet available in the literature for FLB. UPLC is accepted as new liquid chromatography. UPLC is defined as "speed, resolution and sensitivity" by 'Waters' that the first manufacturer of the UPLC system.[9]

Gel formulations are very important for the pharmaceutical field and provide better application and stability when compared to creams and ointments.[10] Topical gel medication is a localized drug delivery system anywhere on the body, via ophthalmic, rectal, vaginal and topical routes via the skin. The skin is one of the most common and easily accessible organs in the human body for topical application and is the main route of topical drug applications.[10,11] Because of its non-toxic properties, cellulose derivatives are used as emulsifiers, colloidal stabilizers and gel agents in pharmaceutical and food industries.[11] The methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) are used for model development and are thermosensitive physical hydrogels. MC is a water-soluble polymer commonly used as a thickener or binder in pharmaceutical, ceramic processing and food applications. HPMC like MC, is used as a hydrophilic carrier material in a wide variety of food and drug applications, especially in oral controlled drug delivery systems.[12] Carbopol® 940 (C-940), a synthetic polymer, has recently been used frequently as part of drug delivery systems. Since the rheological properties are usually investigated by the continuous shear technique which can deform the gel structure, the obtained data does not represent intact gel structure.[13] C-940 is a hydrophilic polyacrylic acid polymer and the carboxyl groups are highly ionized after neutralization, forming a gel due to the electrostatic compression between the charged polymer chains. The most important point in gels prepared with

C-940 is that it prevents the skin from escaping from the environment, causing the hydration of the stratum corneum. This leads to intracellular and intercellular channels and "opening" of the pathway for easier passage of drug molecules.[14]

In this study, a new UPLC method for FLB, which was not previously entered into the literature, has been developed and validated. FLB loaded MC, HPMC, C-940 based gels were prepared and characterized for physical appearence, pH, rheology, drug content, dissolution study and release kinetics study with DD-Solver software program. The UPLC method developed in this study was used for drug amount and dissolution study.

METHODOLOGY

Materials

FLB was obtained from Sanovel (İstanbul/Turkey) as a gift sample. All the other chemicals and reagents used were of analytical grade.

Method development of FLB by UPLC

26 methods (Method 1 to 26) with varying parameters were tested for best resolution, peak shape and minimum & accetable retention time at every single day for the condition of the device. Table 1 gives the UPLC parameters for each method and Table 2 shows the UPLC methodology applied for selected method.

Table 1. UPLC method development studies

Method	Mobile phase composition	Ratio	Flow rate	Rt (min)	Peak morphology
Method 1	Acetonitrile: Methanol	70:30	0.5 mL.dk^{-1}	0.5	Sharp peak
Method 2	Acetonitrile: Methanol	50:50	0.5 mL.dk^{-1}	0.6	Sharp peak
Method 3	Acetonitrile: Methanol: Water	10:70:30	0.5 mL.dk^{-1}	0.5	Spread peak
Method 4	Methanol: Water: Acetic acid %5	65:35:2	0.5 mL.dk^{-1}	1.2	Tailed peak
Method 5	Acetonitrile: Methanol	30:70	0.5 mL.dk^{-1}	0.1	Spread peak
Method 6	Acetonitrile: Buffer 1	50:50	0.5 mL.dk^{-1}	0.4	Sharp peak
Method 7	Methanol: Buffer 1	50:50	0.5 mL.dk^{-1}	0.5	Tailed peak
Method 8	Acetonitrile: Buffer 1	30:70	0.5 mL.dk^{-1}	0.5	Spread peak
Method 9	Acetonitrile: Methanol: Buffer 1	15:15:70	0.5 mL.dk^{-1}	3.0	Spread peak
Method 10	Acetonitrile: Methanol: Buffer 1	15:15:70	0.3 mL.dk^{-1}	5.0	Tailed peak
Method 11	Acetonitrile: Methanol: Buffer 1	30:10:60	0.5 mL.dk^{-1}	0.7	Sharp peak

Method 12	Acetonitrile: Methanol: Buffer 1	20:10:70	0.5 $mL.dk^{-1}$	1.7	Tailed peak
Method 13	Acetonitrile: Buffer 1	20:80)	0.5 $mL.dk^{-1}$	3.0	Spread peak
Method 14	Acetonitrile: Methanol: Buffer 1	20:20:60	0.5 $mL.dk^{-1}$	1.0	Spread peak
Method 15	Acetonitrile: Methanol: Buffer 1	15:20:65	0.5 $mL.dk^{-1}$	2.0	Tailed peak
Method 16	Acetonitrile: Methanol: Buffer 1	15:30:55	0.5 $mL.dk^{-1}$	1.0	Sharp peak
Method 17	Acetonitrile: Methanol: Buffer 1	15:25:60	0.5 $mL.dk^{-1}$	1.5	Sharp peak
Method 18	Acetonitrile: Methanol: Buffer 1	15:15:70	0.6 $mL.dk^{-1}$	2.0	Spread peak
Method 19	Acetonitrile: Methanol: Buffer 1	25:15:60	0.4 $mL.dk^{-1}$	1.0	Tailed peak
Method 20	Acetonitrile: Methanol: Buffer 2	20:20:60	0.5 $mL.dk^{-1}$	3.0	Tailed peak
Method 21	Acetonitrile: Methanol: Buffer 2	30:20:50	0.5 $mL.dk^{-1}$	0.7	Tailed peak
Method 22	Acetonitrile: Methanol: Buffer 2	20:30:50	0.5 $mL.dk^{-1}$	0.4	Tailed peak
Method 23	Acetonitrile: Methanol: Buffer 2	30:10:60	0.3 $mL.dk^{-1}$	1.0	Sharp peak
Method 24	Acetonitrile: Methanol: Buffer 1	30:20:50	0.3 $mL.dk^{-1}$	1.7	Sharp peak
Method 25	Acetonitrile: Methanol: Buffer 1	30:20:50	0.1 $mL.dk^{-1}$	0.8	Spread peak
Method 26	Acetonitrile: Methanol: Buffer 1	30:30:40	0.2 $mL.dk^{-1}$	2.0	Sharp peak

***Rt:** Retention time (minute), ***Buffer 1:** 30 mM disodium hydrogen phosphate buffer,
***Buffer 2:** 0.05 M Potassium dihydrogen phosphate buffer

Table 2. Summary conditions of the UPLC method

Device	Agilent Technology 1290 Infinity
Column	Zorbax Eclipse Plus C18 (2.1x50 mm, 1.8 µm)
Mobile phase	30:30:40 (v/v/v) acetonitrile:methanol: 30 mM disodium hydrogen phosphate buffer
Oven temperature	40°C
Flow rate	0.2 $mL.min^{-1}$
İnjection volume	0.5µL
Wavelength	247

UPLC device (Agilent Technology 1290 Infinity) used was mounted with reversed-phase (RP) Zorbax® Eclipse Plus C18 gravity column (column length: 50 mm, column diameter: 2.1 mm, particle diameter: 1.8 µm). 30:30:40 (v/v/v) acetonitrile: methanol: 30 mM disodium hydrogen phosphate buffer was used as the mobile phase for perfect resolution of FLB. Flow rate of the mobile phase was set to 0.2 $mL.min^{-1}$ and 0.5µL invariable volume of specimen were injected by an automatic injector. Temperature of the column was set to 40°C while a fluorescent detector was used at 247 nm.

Method validation of FLB by UPLC

Linearity and Range

Linearity is a common study used to check the linearity of a calibration curve by examining the correlation coefficient.[15] Aliquots from a standard stock solution (250 $\mu g \cdot mL^{-1}$) of FLB were used to prepare different sets of dilutions. A series of dilutions consisted of 10 different concentrations of FLB in the range of 5-50 $\mu g \cdot mL^{-1}$. Absorbance values were measured and calculations were made to determine FLB concentration. The specified range is derived from linearity studies and depends on the intended application of the procedure.16 Therefore, a standard stock solution (250 $\mu g \cdot mL^{-1}$) of FLB were used to prepare in the range of 5-250 $\mu g \cdot mL^{-1}$.

Specificity

The specificity of the UPLC method was determined by complete separation of the FLB with the mobile phase, pH 7.4 buffer and then the effect of the excipients used in the gel formulation was investigated with placebo formulations to determine whether or not they have been interfered.

Precision

Precision is an extremely important criterion for all analysis that exhibits "closeness to agreement" between a set of measurements.[17] Intermediate precision and repeatability values when using the device in this study was verified by repeated scanning and measurement of absorbances (n=6) for FLB (15 $\mu g \cdot mL^{-1}$, 30 $\mu g \cdot mL^{-1}$, 45 $\mu g \cdot mL^{-1}$). Repeatability studies were performed six times on the same day by analyzing three different concentrations of 15 $\mu g \cdot mL^{-1}$, 30 $\mu g \cdot mL^{-1}$, 45 $\mu g \cdot mL^{-1}$ for FLB. Repeating tests on three consecutive days verified intermediate precision of the method. Results were expressed as RSD% of the measurements obtained.

Limit of detection and limit of quantitation (sensitivity)

Detection and quantification limits are the two principal components of method validation [18]. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were

separately determined based on the calibration curve obtained according to ICH Q2 (R1) recommendations (Eq. 1, Eq. 2). Standard deviation of y-intercept and slope of the calibration curve were used to calculate LOD and LOQ, respectively.

$$LOD = 3.3 \times \sigma/S \qquad \text{Equation 1}$$

$$LOQ = 10 \times \sigma/S \qquad \text{Equation 2}$$

where, σ = the standard deviation of the response and S = slope of the calibration curve.

Accuracy

Accuracy was calculated as deviation of mean from nominal concentration.[19] Accuracy of the method used was determined by calculating recoveries of FLB by standard addition method. Standard solutions containing specific amount of FLB (20 μg•mL^{-1}, 30 μg•mL^{-1}, 40 μg•mL^{-1}) were used and percentage of recoveries were calculated.

Robustness

Robustness is the measure of the analytical method's ability to remain unaffected by small changes in method parameters. The factors chosen for this Robustness study were the wavelength (nm), temperature (◦C), flow (mL.min−1), pH of mobile phase. The factors are shown in Table 3.

Table 3. Experimental design of the robustness study

No	Wavelength (nm)	Temperature (°°C)	Flow rate (ml. min^{-1})	pH of mobile phase
1	247	37	0.20	7.4
2	247	37	0.18	7.8
3	245	40	0.18	7.8
4	245	40	0.20	7.4
5	247	40	0.18	7.4
6	245	37	0.20	7.8

Preparation of gel formulations

The composition of FLB topical gel formulations are shown in Table 4 and Table 5. For water-based formulations, the amount of polymer required was weighed and sprinkled on the water surface at about 500 rpm for 2 hours (Solution A). 0.5 g FLB is then dissolved in the appropriate amount of alcohol, glycerin (GLY) and propylene glycol (PG) (Solution B). Finally, solution B was added into solution A under magnetic stirring. These two mixtures were further stirred under continuous stirring for 2 hours.

For dimethyl sulfoxide (DMSO) based formulations, the amount of polymer required was weighed and sprinkled on the DMSO surface at about 500 rpm for 2 hours (Solution C). 0.5 g FLB is then dissolved in the appropriate amount of alcohol, glycerin and propylene glycol (Solution D). Finally, solution D was added into Solution C under stirring. These two mixtures were further stirred under continuous stirring for 2 hours. The gel formulations prepared were filled into aluminum collapsible tubes for characterization studies, folded and sealed.

Table 4. Gel formulations prepared with distilled water

Code	MC (g)	HPMC (g)	C-940 (g)	GLY (g)	PG (g)	D. water (g)	Alcohol (g)	FLB (g)
A-1	0.200	-	-	1.000	1.000	5.300	2.000	-
A-2	0.250	-	-	1.000	1.000	5.250	2.000	-
A-3	0.300	-	-	1.000	1.000	5.200	2.000	-
A-4	0.400	-	-	1.000	1.000	5.100	2.000	-
A-5	0.500	-	-	1.000	1.000	5.000	2.000	-
A-6	0.200	-	-	1.000	1.000	5.300	2.000	0.500
A-7	0.250	-	-	1.000	1.000	5.250	2.000	0.500
A-8	0.300	-	-	1.000	1.000	5.200	2.000	0.500
A-9	0.400	-	-	1.000	1.000	5.100	2.000	0.500
A-10	0.500	-	-	1.000	1.000	5.000	2.000	0.500
B-1	-	0.200	-	1.000	1.000	5.300	2.000	-
B-2	-	0.250	-	1.000	1.000	5.250	2.000	-
B-3	-	0.300	-	1.000	1.000	5.200	2.000	-
B-4	-	0.400	-	1.000	1.000	5.100	2.000	-
B-5	-	0.500	-	1.000	1.000	5.000	2.000	-
B-6	-	0.200	-	1.000	1.000	5.300	2.000	0.500
B-7	-	0.250	-	1.000	1.000	5.250	2.000	0.500
B-8	-	0.300	-	1.000	1.000	5.200	2.000	0.500
B-9	-	0.400	-	1.000	1.000	5.100	2.000	0.500
B-10	-	0.500	-	1.000	1.000	5.000	2.000	0.500
C-1	-	-	0.040	1.000	1.000	5.960	2.000	-
C-2	-	-	0.050	1.000	1.000	5.950	2.000	-
C-3	-	-	0.100	1.000	1.000	5.900	2.000	-
C-4	-	-	0.150	1.000	1.000	5.850	2.000	-
C-5	-	-	0.200	1.000	1.000	5.800	2.000	-
C-6	-	-	0.040	1.000	1.000	5.960	2.000	0.500
C-7	-	-	0.050	1.000	1.000	5.950	2.000	0.500
C-8	-	-	0.100	1.000	1.000	5.900	2.000	0.500
C-9	-	-	0.150	1.000	1.000	5.850	2.000	0.500
C-10	-	-	0.200	1.000	1.000	5.800	2.000	0.500

*g: gram

Table 5. Gel formulations prepared with DMSO

Code	MC (g)	HPMC (g)	C-940 (g)	GLY (g)	PG (g)	DMSO (g)	Alcohol (g)	FLB (g)
D-1	0.200	-	-	2.000	2.000	2.300	3.000	-
D-2	0.250	-	-	2.000	2.000	2.250	3.000	-
D-3	0.300	-	-	2.000	2.000	2.200	3.000	-
D-4	0.400	-	-	2.000	2.000	2.100	3.000	-
D-5	0.500	-	-	2.000	2.000	2.000	3.000	-
D-6	0.200	-	-	2.000	2.000	2.300	3.000	0.500
D-7	0.250	-	-	2.000	2.000	2.250	3.000	0.500
D-8	0.300	-	-	2.000	2.000	2.200	3.000	0.500
D-9	0.400	-	-	2.000	2.000	2.100	3.000	0.500
D-10	0.500	-	-	2.000	2.000	2.000	3.000	0.500
E-1	-	0.200	-	2.000	2.000	2.300	3.000	-
E-2	-	0.250	-	2.000	2.000	2.250	3.000	-
E-3	-	0.300	-	2.000	2.000	2.200	3.000	-
E-4	-	0.400	-	2.000	2.000	2.100	3.000	-
E-5	-	0.500	-	2.000	2.000	2.000	3.000	-
E-6	-	0.200	-	2.000	2.000	2.300	3.000	0.500
E-7	-	0.250	-	2.000	2.000	2.250	3.000	0.500
E-8	-	0.300	-	2.000	2.000	2.200	3.000	0.500
E-9	-	0.400	-	2.000	2.000	2.100	3.000	0.500
E-10	-	0.500	-	2.000	2.000	2.000	3.000	0.500
F-1	-	-	0.040	2.000	2.000	2.960	3.000	-
F-2	-	-	0.050	2.000	2.000	2.950	3.000	-
F-3	-	-	0.100	2.000	2.000	2.900	3.000	-
F-4	-	-	0.150	2.000	2.000	2.850	3.000	-
F-5	-	-	0.200	2.000	2.000	2.800	3.000	-
F-6	-	-	0.040	2.000	2.000	2.960	3.000	0.500
F-7	-	-	0.050	2.000	2.000	2.950	3.000	0.500
F-8	-	-	0.100	2.000	2.000	2.900	3.000	0.500
F-9	-	-	0.150	2.000	2.000	2.850	3.000	0.500
F-10	-	-	0.200	2.000	2.000	2.800	3.000	0.500

*g: gram

Gel characterization studies

Physical appearence

The prepared gel formulations were inspected visually for their colour and homogeneity.

pH

The pH of the FLB loaded gels were determined using digital pH meter (Mettler Toledo™ S220 SevenCompact™ pH/lon Benchtop Meter). The measurements were taken for average of 3 times.

Rheological Characterization

Rheological properties were determined using a cone-and-plate geometry rheometer with a diameter of 40 mm (Brookfield, USA). Measurements and viscosity changes were repeated at 25 ± 1°C temperatures. Shear rates against shear stress were calculated. Measurements provide further information about flow properties.

Drug content

Fully weighed 1 g of gel was removed and dissolved in 100 mL of pH 7.4 phosphate buffer. The volumetric flask containing the gel solution was agitated for 2 hours on a mechanical shaker to obtain the complete solubility of the drug. This solution was filtered using a Millipore filter (0.45 μm). After appropriate dilution, it was analyzed by the developed UPLC method. Measurements were repeated three times.

Dissolution study

In vitro release study of the gel formulations was investigated for 6 hours time. *In vitro* drug release of FLB from gel formulations were studied through dialysis bag (cellulose membrane) which was sealed with clamps and stirred at 250 rpm using magnetic stirrer. The temperature was maintained at 37 ± 0.5°C under sink conditions. Gel formulation equivalent to 0.2 g of drug and 0.2 g pure FLB were transferred into dialysis membrane which was previously soaked in dissolution medium for 12 hours, tied properly at both the ends and kept inside the glass. The *in vitro* release studies were performed in phosphate buffer (pH 7.4).[20] Samples were collected at certain intervals from the release media and the same volume was completed with a fresh dissolution medium. The samples were then analysed by a developed and validated UPLC method.

Release kinetics

To determine the release kinetics, data obtained from in vitro drug release studies in phosphate buffer (pH 7.4) were analyzed by a software program DDSolver.[21]

RESULTS AND DISCUSSION

Method development and validation of FLB by UPLC

Different proportions of acetonitril:methanol:30mM disodium hydrogen phosphate buffer and acetonitril: methanol:0.05M Potassium dihydrogen phosphate buffer and flow rates were tested for method optimization and it was found that acetonitril: methanol:30 mM disodium hydrogen phosphate buffer the proportion of 30:30:40 v/v/v and a flow rate of 0.2 $mL.min^{-1}$ give admissible retention time (RT) and good resolution for both the mobile phase, placebo formulations, pH 7.4 buffer and FLB.

Linearity and Range

Linearity range of FLB for the method used was found to be 5-50 $\mu g \cdot mL^{-1}$ while regression equation was determined to be y=152920x-206333 by plotting concentration (x) versus peak area (y). Correlation coefficient (R^2) of 0.9999 was highly significant. Linearity test results are shown in Table 6 and regression curve is presented in Figure 1.

Table 6. Series and area values prepared for linearity study

CONC ($\mu g.mL^{-1}$)	Area/Rt					
	SET 1	SET 2	SET 3	Mean	SD	SE
5.0	61.7205	59.4184	58.3159	59.8183	1.7372	0.7092
10.0	132.6282	130.8683	134.4335	132.6433	1.7826	0.7278
15.0	203.9623	206.8451	206.6789	205.8288	1.6185	0.6608
20.0	285.7185	283.5822	281.8051	283.7019	1.9594	0.7999
25.0	363.9279	360.1898	358.7766	360.9648	2.6617	1.0866
30.0	439.5623	440.0372	433.7357	437.7784	3.5092	1.4326
35.0	520.3603	509.4565	510.3425	513.3864	6.0557	2.4722
40.0	590.8665	591.0432	587.2816	589.7304	2.1226	0.8665
45.0	672.6392	666.9029	668.4010	669.3143	2.9752	1.2146
50.0	753.8879	748.1176	735.3897	745.7984	9.4647	3.8639
75.0	1113.503	1116.422	1158.8123	1129.579	25.3588	10.3527
90.0	1368.832	1380.642	1371.7079	1373.727	6.1587	2.5143
120.0	1799.558	1824.454	1853.7453	1825.919	27.1236	11.0731
150.0	2230.738	2266.369	2309.2433	2268.784	39.3083	16.0476
200.0	3003.514	3039.887	3063.1810	3035.528	30.0712	12.2765
250.0	3808.190	3792.207	3856.2010	3818.866	33.3061	13.5972

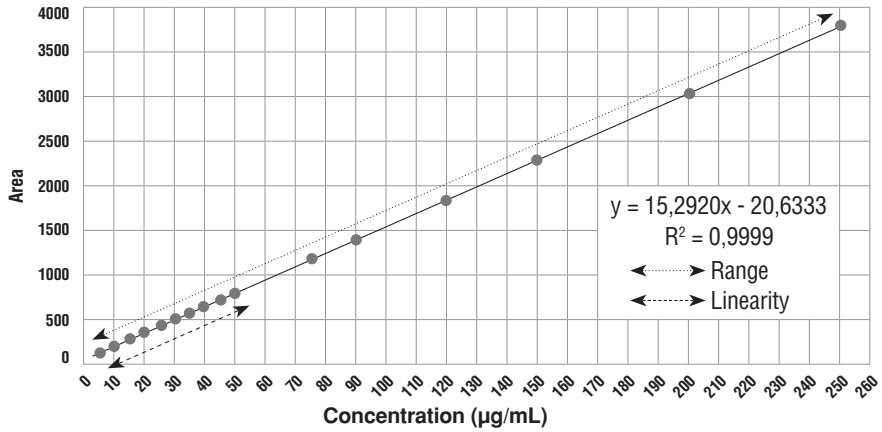

Figure 1. Regression profile of FLB.

Range is the interval between the upper and lower concentration of active agent that have been indicated to be determined with precision, accuracy and linearity using the method as written.[22] The accuracy and precision of the method are within the acceptable range. In this study the range was observed linearly to the highest concentration (250 µg.mL $^{-1}$, R2: 0.9999).

Specificity and peak morphology

Characteristic UPLC chromatogram of FLB is given at Figure 2. It can be seen that chromatogram recorded for the combination of non-functioning components exposed no peaks at retention time of 2.0 minutes (Figure 3 and Figure 4).

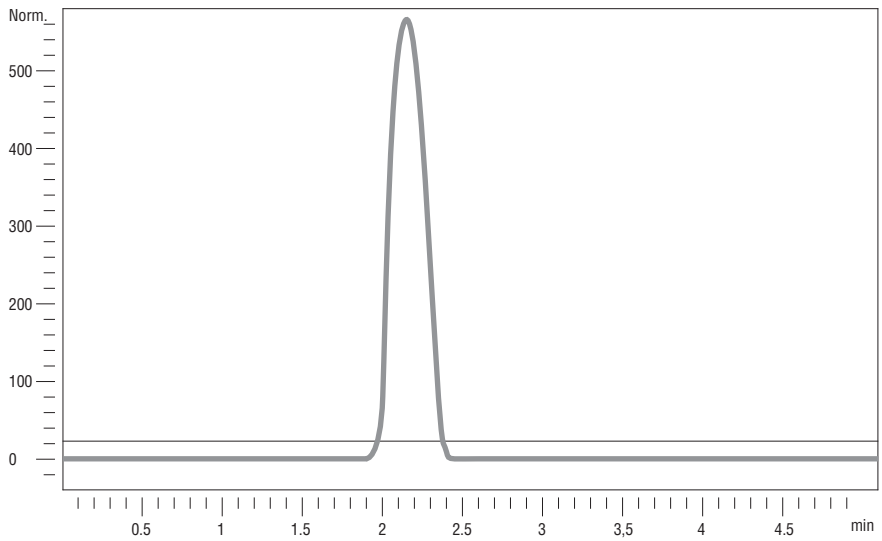

Figure 2. Chromatogram of FLB

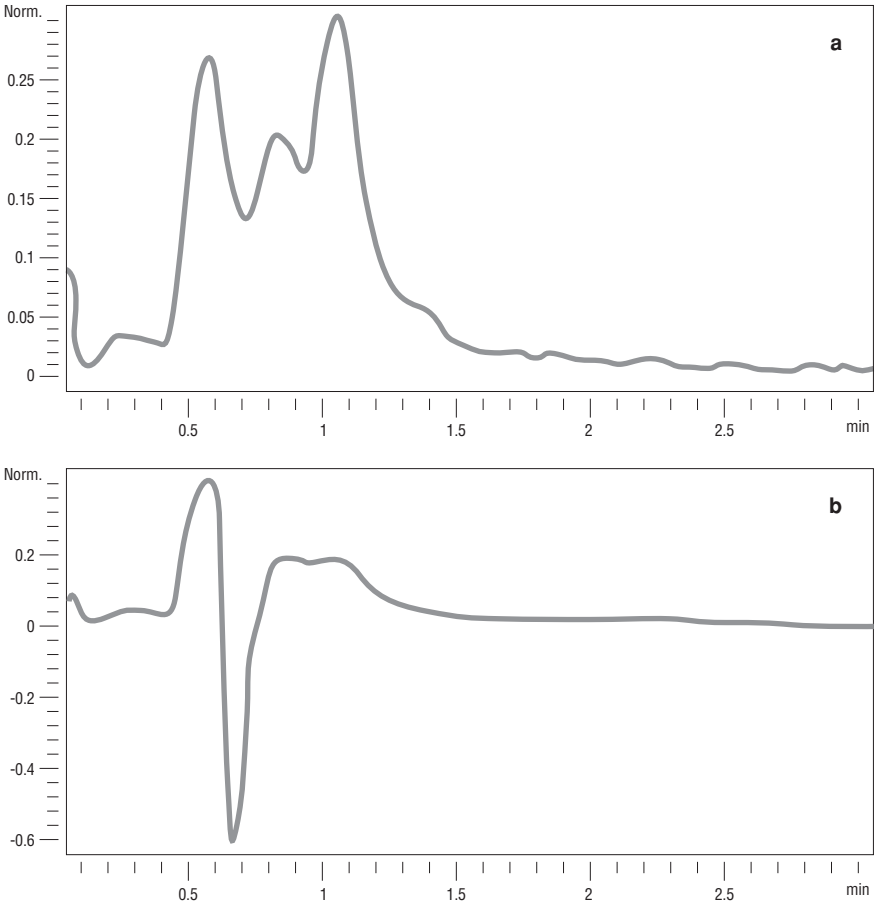

Figure 3. Chromatogram of Mobile phase and pH 7.4 phosphate buffer **a:** mobile phase **b:** pH 7.4 phosphate buffer

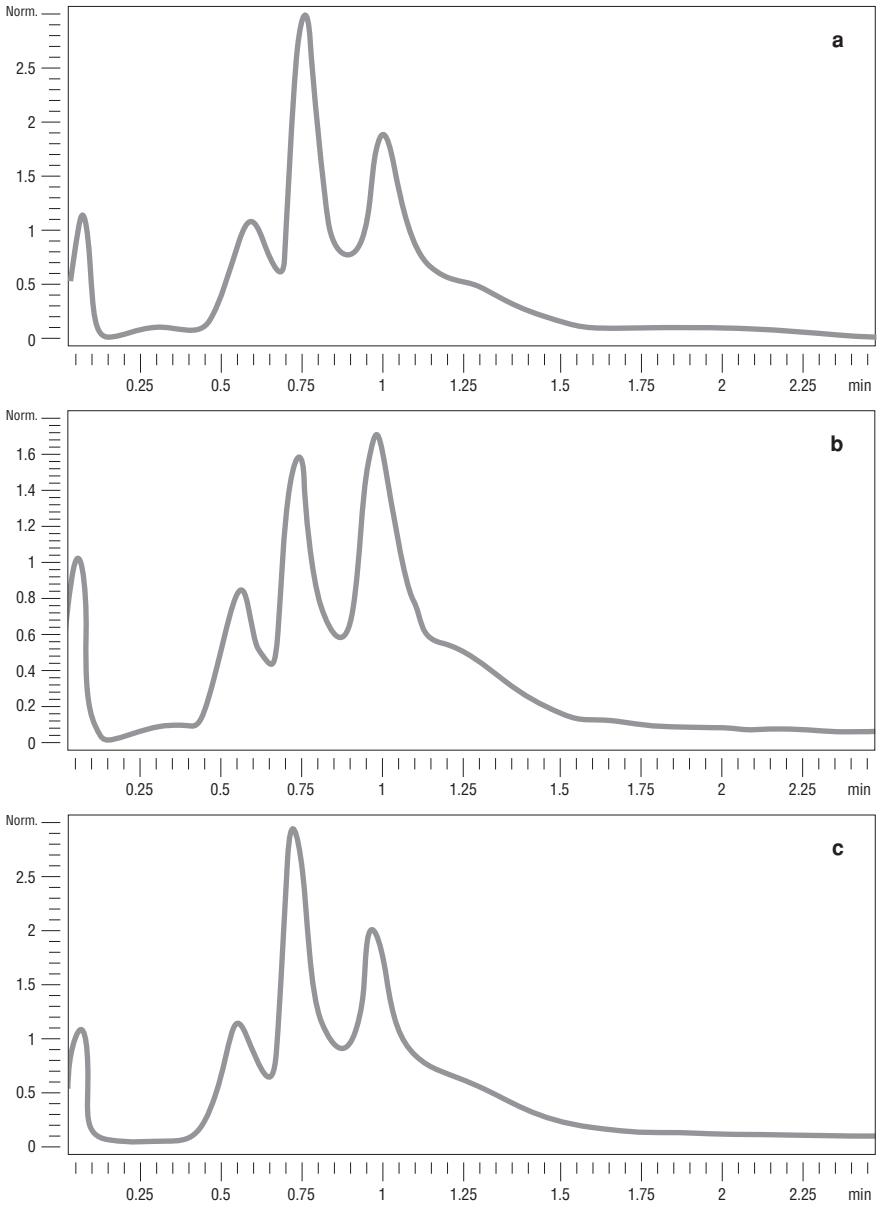

Figure 4. Chromatogram of placebo formulations a: D-2 b: E-1 c: F-4

Precision

Results of intermediate precision and repeatability tests on different concentrations are given in Table 7. RSD values for both intermediate precision and repeatability were <2 %. Therefore, the method developed for FLB was found to be precise according to the suggestions in ICH Q2(R1) guidelines and also in literature.[23]

Table 7. Precision results for 15 µg.mL^{-1} 30 µg.mL^{-1} 45 µg.mL^{-1} of FLB

Area/Rt			Concentration (15 µg.mL^{-1})		
1st day	**2nd day**	**3rd day**	**1st day**	**2nd day**	**3rd day**
218.4481	206.6694	218.7202	15.6344	14.8642	15.6522
220.0384	205.5260	216.8623	15.7384	14.7894	15.5307
213.0078	203.1071	213.5887	15.2787	14.6312	15.3166
Mean	15.5505	14.7616	15.4999		
Standard deviation (SD)			0.2411	0.1189	0.1699
Coefficient of variation (RSD)			1.5504	0.8057	1.0961
95 % confidence interval			0.5989	0.2995	0.4220
Area/Rt			**Concentration (30 µg.mL-1)**		
1st day	**2nd day**	**3rd day**	**1st day**	**2nd day**	**3rd day**
452.4530	440.2308	460.0505	30.9369	30.1376	31.4337
435.4347	455.5667	445.2264	29.8240	31.1405	30.4643
444.1183	451.2570	445.6063	30.3918	30.8587	30.4891
Mean	30.3842	30.7122	30.7957		
Standard deviation (SD)			0.5565	0.5172	0.5527
Coefficient of variation (RSD)			1.8315	1.6841	1.7946
95 % confidence interval			1.3824	1.2848	1.3729
Area/Rt			**Concentration (45 µg.mL^{-1})**		
1st day	**2nd day**	**3rd day**	**1st day**	**2nd day**	**3rd day**
452.4530	440.2308	460.0505	30.9369	30.1376	31.4337
435.4347	455.5667	445.2264	29.8240	31.1405	30.4643
444.1183	451.2570	445.6063	30.3918	30.8587	30.4891
Mean	30.3842	30.7122	30.7957		
Standard deviation (SD)			0.5565	0.5172	0.5527
Coefficient of variation (RSD)			1.8315	1.6841	1.7946
95 % confidence interval			1.3824	1.2848	1.3729

Limit of detection and limit of quantitation (sensitivity)

Analytical method development and validation procedures are very important for the discovery and development of drugs. LOD and LOQ parameters are interrelated, but have different definitions and should not be confused. There are a few definitions used to describe LOD and LOQ. In general, an LOD is detected as the lowest concentration in a sample under the conditions specified in the test, but is not considered to be quantifiable. LOQ is the lowest concentration of an analyte in a test and can be determined with acceptable precision and accuracy under the specified test conditions. There are several common methods for estimating

the detection and quantification limit, which can be listed as follows; signal-to-noise, blank determination, linear regression, limit of blank and precision-based approaches.[24,25] In this study, LOD and LOQ were calculated by linear regression and found as 0.0607 µg.mL-1 and 0.1840 µg.mL^{-1}, respectively. The linear regression method used in this study can be applied in every situation and the analysis method is most suitable if it does not contain noise in the background. The calibration curve uses a series of low values close to zero and results in a more relevant evaluation with a more homogeneous distribution.[25]

Accuracy

As shown in Table 8 perfect recoveries of FLB at various concentrations were obtained between 100.3863 - 101.0911% and also RSD values for all concentration were <2 %.25,26 Table 8 indicates good accuracy of the UPLC method developed in this study.

Table 8. Accuracy results for 20 µg.mL-1 30 µg.mL-1 40 µg.mL-1 of FLB

Area/Rt			**Concentration**		
20 µg.mL^{-1}	**30 µg.mL^{-1}**	**40 µg.mL^{-1}**	**20 µg.mL^{-1}**	**30 µg.mL^{-1}**	**40 µg.mL^{-1}**
287,4006	438,4561	598,9059	20,1435	30,0216	40,5140
285,9462	441,4241	595,5505	20,0484	30,2156	40,2945
286,4823	439,8155	598,7052	20,0834	30,1105	40,5008
			Recovery %		
			20 µg.mL^{-1}	**30 µg.mL^{-1}**	**40 µg.mL^{-1}**
			100,7174	100,0718	101,2849
			100,2418	100,7188	100,7363
			100,4171	100,3682	101,2521
Recovery % (mean)			100.4588	100.3863	101.0911
Difference %			0.4588	0.3863	1.0911
Standard deviation			0.2405	0.3239	0.3077
Coefficient of variation (RSD)			0.2394	0.3226	0.3044
Standart Error			0.0982	0.1322	0.1256
95 % confidence interval			0.5974	0.8045	0.7643

Robustness

Results were obtained for area response and retention time, % RSD was calculated and examined for robustness. % RSD for retention time for six different condutions were between 0.20 and 0.76 % (Table 9), which is is well inside the proposed acceptance basis of ≤5 %. Percent RSD for area response was from 0.09 to 0.73 %, which also passed the proposed acceptance basis of ≤2 %.[26,27,28] Therefore, it can be concluded that the method is consistent in front of the wavelength, temperature, flow and pH of mobile phase.

Table 9. %RSD for robustness study (n=6)

No	%RSD, Retention Time	%RSD, Peak Area
1	0.20	0.09
2	0.63	0.73
3	0.76	0.73
4	0.63	0.68
5	0.72	0.24
6	0.28	0.11

Gel characterization studies

Physical appearence

All gel formulations showed good homogeneity in the absence of pellets. Their color was determined to be transparent both in the placebo and in the active ingredient formulations.[29,30]

pH

pH results of prepared gel formulations were shown in Table 10. The pH of all gel formulations were found near to the skin pH, that showed the gels were suitable for topical delivery.[31]

Table 10. Result of pH and drug content

Code	pH	Drug content (%)
D-7	5.79±0.26	98.14±0.06
E-6	5.82±0.21	98.21±0.04
F-9	5.71±0.39	99.02±0.02

*all result gives with standart error

Rheological Characterization

Rheological measurements were performed for carbopol, MC and HPMC gels. The results of these measurements are presented in Figure 5. Flow index provides an idea about the flow properties of the formulation from the container.

All gels are clearly shear thinning fluids and have the tendency to become non-Newtonian at low shear rates. The pseudoplasticity is because of the gelling structure, which lead to decrease in viscosity with increase in shear rate.[32,33] Because of the pseudoplastic flow, the gel system will require application of some force to take. The sheer stress/ shear rate datas for Casson Model were compatible with the literature.[32] Flow curves obtained at room temperatures indicate gels show

significant pseudoplastic behavior with a Casson Model ranging from 10 to 20 s^{-1} (Figure 5). Non-Newtonian flow properties in gel formulations can be found to the increased solvent-solvent and polymer-solvent attractions and higher viscosity of cosolvent.[33,35]

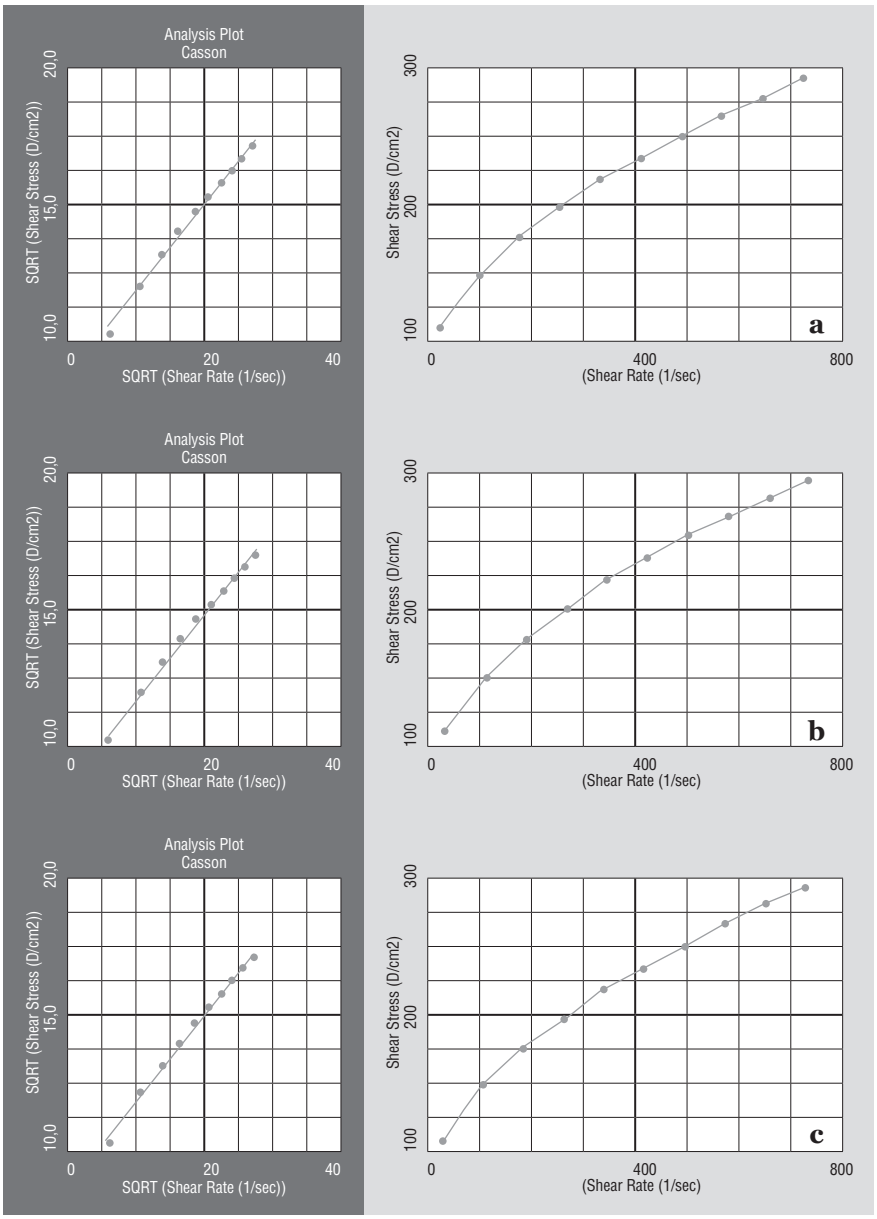

Figure 5. Rheological measurements results **a:** D-7 **b:** E-6 **c:** F-9

Drug content

In the prepared gel formulations, the drug content was found to be in the range of 98.14-99.02 % indicating the uniformity of the high drug content.[35,36] The drug content results of the gel formulations are shown in Table 10.

Dissolution study

In vitro dissolution profile of FLB gels containing different gelling agent are shown in Figure 6. The initial concentration of FLB in all gel formulations was kept constant at 0.2 grams. It was determined that the release rate of pure FLB reached 100% within 2 hours. At the end of the 6th hour, the FLB release from gels prepared with MC (D-7) and HPMC (E-6) reached 99.7 % and 99.5 % respectively. The release rate of the gel prepared with C-940 (F-9) was 87.60%. Viscosity is negatively related to the formulation release of the active ingredients and their penetration through diffusion barriers. The reduction in release can be attributed to the excess viscosity of the F-7 coded formulation over the other two formulations.35 In the light of these results, it was observed that the polymer type and viscosity was the most affected factor in releasing the drug.[36] At the same time the results suggest that formulations prepared according to pure FLB have prolonged release.

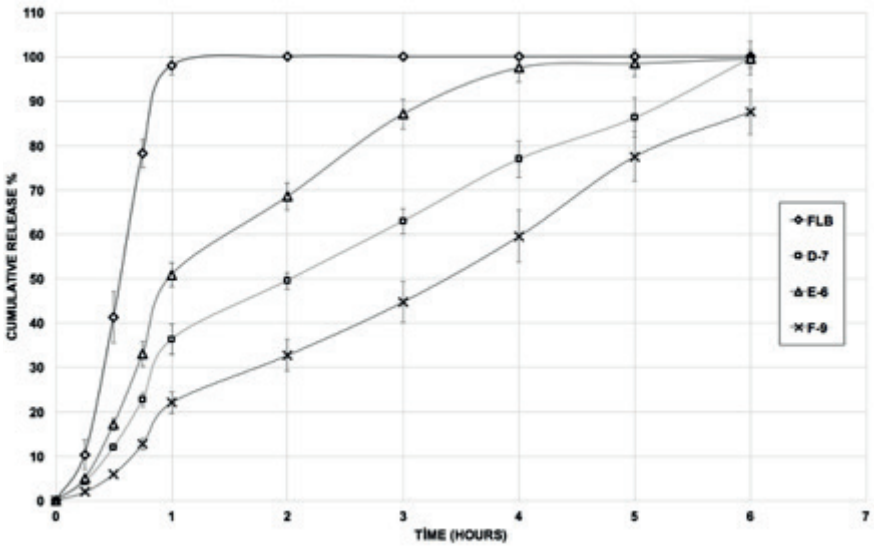

Figure 6. *In vitro* release of pure FLB and FLB loaded gels

Release kinetics

Dissolution testing is a crucial analysis for both drug research development and quality control because it determines the rate and extent of drug release from oral pharmaceutical products. Dissolution data analysis is determined by statistically or mathematically comparing the dissolution profiles to quantify or characterize drug release from a pharmaceutical formulation.[21] Almost all of the commercial statistical software programs used on the pharmaceutical field are designed for evaluating pharmacokinetic parameters (*in vivo* study), not for statistical evaluation of dissolution profiles (*in vitro* dissolution study). To reduce computation time and eliminate computational errors, researchers designed the DDsolver program, an excel add-in program that allows modeling of dissolution data using a different dissolution model. The program provides an efficient data analysis report to summarize the analysis of the dissolution data.[21,37] In this study, different kinetic models were applied on release data for categorizing the kinetics of drug release with DDSolver computer program. This program was used to shorten the calculation time, to eliminate calculation errors and to determine the correct release profile. When all optimum gel formulations were analyzed for cumulative solubility in time versus time, all formulations appeared to be continuously released for 6 hours. After calculation, the data is transferred to the DDSolver program to determine five important and the most popular criteria. These criteria are based on the coefficient of determination (Rsqr, R^2, or COD), the adjusted coefficient of determination (Rsqr_adj or $R^2_{adjusted}$), the Akaike Information Criterion (AIC), the Model Selection Criterion (MSC) and n for only korsmeyer peppas models. The highest R^2, $R^2_{adjusted}$ and MSC values and the lowest AIC values are used for the evaluation.[21,38] Zero-order kinetic, First-order kinetic, Higuchi, Korsmeyer-Peppas, Korsmeyer-Peppas with T_{lag}, Korsmeyer-Peppas with F_o and Weibull models were selected for evaluation in DDSolver program. As a result of applying in vitro release study data obtained to different kinetic models using DDSolver program; R^2, $R^2_{adjusted}$ MSC, AIC found are shown in Table 11.

Table 11. Kinetic modeling of gel formulation by DDSolver program

Code	Model and Equation	Evaluation Criter				
		R2	R2 adjusted	AIC	MSC	n
D-7	Zero-order model* $F=k_0*t$	0.914	0.914	62.067	2.226	-
E-6		0.686	0.686	75.256	0.935	-
F-9		0.990	0.990	41.027	4.404	-
D-7	First-order model* $F=100*[1-Exp(-k_1*t)]$	0.812	0.812	69.051	1.450	-
E-6		0.974	0.974	52.874	3.422	-
F-9		0.948	0.948	56.083	2.728	-
D-7	Higuchi model* $F=k_H*t^{0.5}$	0.934	0.934	59.633	2.496	-
E-6		0.920	0.920	62.917	2.306	-
F-9		0.859	0.859	64.982	1.739	-
D-7	Korsmeyer-Peppas* $F=k_{KP}*t^n$	0.910	0.897	64.469	1.959	0.915
E-6		0.882	0.865	68.451	1.691	0.431
F-9		0.970	0.966	52.984	3.072	0.956
D-7	Korsmeyer-Peppas with Tlag model* $F=k_{KP}*(t-T_{lag})^n$	0.939	0.919	62.893	2.134	0.820
E-6		0.864	0.819	71.692	1.331	0.389
F-9		0.976	0.968	53.203	3.048	1.003
D-7	Korsmeyer-Peppas with F0 model* $F=F_0+k_{KP}*t^n$	0.845	0.794	71.297	1.200	1.027
E-6		0.752	0.670	77.107	0.729	0.485
F-9		0.904	0.872	65.558	1.675	1.236
D-7	Weibull model* $F=100*\{1-Exp[-((t-Ti)^{\beta})/\alpha]\}$	0.987	0.965	58.015	2.676	-
E-6		0.994	0.987	50.482	3.688	-
F-9		0.990	0.979	51.921	3.190	-

*In all models, *F* is the fraction (%) of drug released in time t, k_0: zero-order release constant, k_1:bfirst-order release constant, k_H: Higuchi release constant, k_{KP}: release constant incorporating structural and geometric characteristics of the drug-dosage form, *n*: is the diffusional exponent indicating the drug-release mechanism, F_0 is the initial fraction of the drug in the solution resulting from a burst release, α: is the scale parameter which defines the time scale of the process; β: is the shape parameter which characterizes the curve as either exponential ($\beta=1$; case 1), sigmoid, S-shaped, with upward curvature followed by a turning point ($\beta>1$; case 2), or parabolic, with a higher initial slope and after that consistent with the exponential ($\beta<1$; case 3), Ti: is the location parameter which represents the lag time before the onset of the dissolution or release process and in most cases will be near zero, T_{lag}: is the lag time prior to drug release.

For gel formulations prepared with cellulose derivatives; the release of FLB from D-7 and E-6 coded formulations was consistent with the Weibull model according to the criterion. The results in this study can be verified on the grounds of previous cellulose derivates studies which demonstrates that researchers mostly emerged

as the most appropriate model for the Weibull model.[39,40,41] From the gel formulation prepared with C-940 (F-9), the release of FLB had zero-order kinetics according to the criterion. This rate of release is preferred because the drug is given in a constant rate for a long time. For the gel formulation prepared with C-940 (F-9), the n value of the Korsymear-Peppas, Korsymear-Peppas with Tlag model and Korsymear-Peppas with F0 model is closer to 1. This information indicates the zero order kinetics.[14] The release kinetic profiles corresponding to all models are automatically extracted from the program and presented in Figure 7.

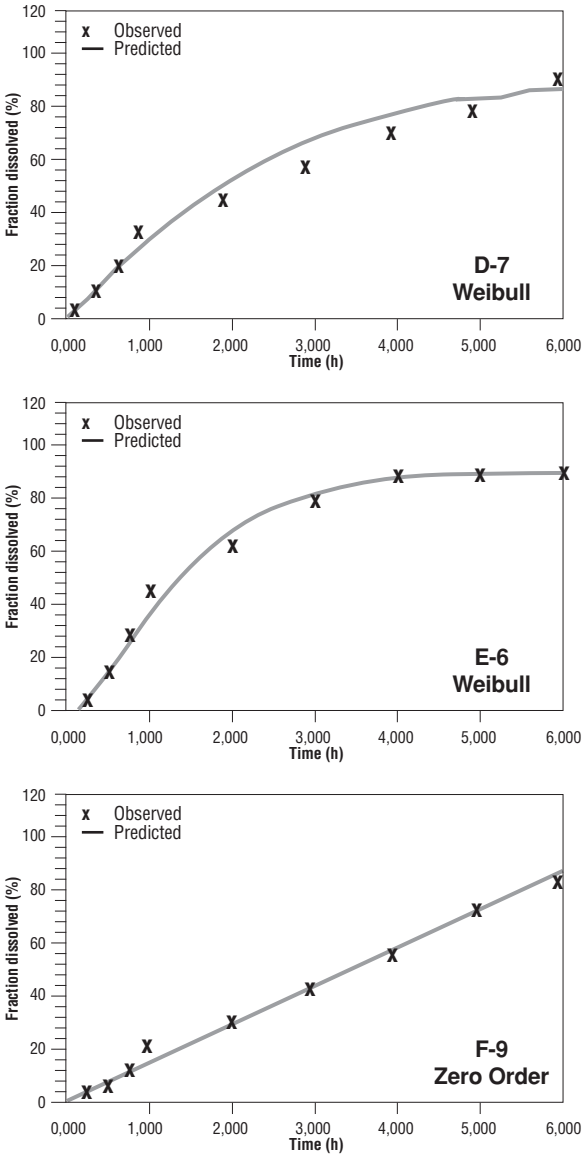

Figure 7. Automated release kinetic profiles of gel formulations from DDSolver software for more suitable model

CONCLUSION

According to the in vitro studies results, a useful methodology for the detection of FLB in topical formulations has been established, which will provide a future basis for the development of a topical dosage form of the drug with a desired release profile. The general rank order of FLB release from the formulations was determined as MC > HPMC > C-940. Gel formulations of HPMC and MC have been observed to give higher values of drug release, which is due to the higher solubility of the drug. Pseudoplastic flows with thixotropy were obtained for all FLB-gels. Thus, these developed systems could be a promising vehicle for topical delivery of FLB. Additionally, in this study also a new, economic, easy and sensitive ultra performance liquid chromatography method was developed for the determination of FLB. The method developed for FLB was decided to be precise due to RSD values of <2% for repeatability and intermediate precision. Recovery of the method was satisfactory owing to <2% RSD value. The drug content was found to be in the range of 98.14-99.02% indicating the uniformity of the high drug content. In the release kinetic tests with DDSolver, the release of gels prepared with methylcellulose and hydroxypropyl methylcellulose showed conformity with the weibull model, whereas the gel formulation prepared with Carbopol® 940 showed a zero-order kinetics. In the case of different variants of similar polymer formulations, it was found that having a higher viscosity with a hyrophylic polymer released a higher amount of drug compared with the carbopol formulations. Further studies will be focused on the *in vivo* animal studies and tissue distribution in order to get a proper insight into the potential of polymeric based gel formulations in topical delivery.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Maternal Monosodium Glutamate Intake Influences the Learning Ability of the Offspring of Sprague Dawley Rats

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ABSTRACT

Objectives: Monosodium glutamate (MSG) is one of the most widely consumed food additives. We aimed to identify the effects of maternal MSG intake on offspring's learning ability in rats.

Patients and Methods: Among thirty female rats, ten control rats were fed by standart diet. Twenty rats were applied 4 mg/g.body weight for 3 weeks and then divided into group I (exposed MSG during pregnancy and lactation) and group II (no MSG). Pups had no MSG at any stage. The adult rats and the pups were applied Barnes maze to test learning ability.

Results: Barnes maze test resulted that the learning ability for offspring of MSG treated mother rats decreased in the order of control > maternal MSG intake for 3 weeks before mating > additional maternal MSG intake during pregnancy and lactation.

Conclusion: In conclusion maternal MSG intake increased the duration of trial and the number of false trial of offspring in Sprague Dawley rats.

Keywords: Monosodium Glutamate, Barnes Maze, Learning Ability, Memory, Sprague Dawley Rats

INTRODUCTION

Monosodium glutamate has been using widely all around the world and contains 78% of glutamic acid, 22% of sodium and water. It is consumed as a flavor enhancer or food additive and used in many commercially packed food and cooking.[1] Glutamic acid takes place in all cells of the body and acts in the intermediary metabolism and as a constituent of the proteins.

Learning is the process that modifies subsequent behavior. Memory is the ability

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to remember past experiences. The application of MSG to rats and mice during the neonatal period caused obesity, learning difficulty, gonadal dysfunction, lesions of neural structures and of the retina.[2-8] Systemic administration of MSG resulted in glutamate-induced cell death in the fetal hypothalamic *neuronal* cell line RCA-6 indicating the defect in specific brain regions, including hypothalamus and cerebellum.[9] Indeed, neurotoxicity of MSG was found to be related with glutamate receptors activation, specifically N-methyl D-aspartate (NMDA) receptors.[10-13] Numerous studies revealed evidences that the hippocampus had a critical role in the processes related to learning and memory in human and animal subjects.[14-16] Barnes maze is one of the common applied maze tests and often employed to assess learning capacity and spatial memory for mice and rats.[17-19]

The oral lethal dose (LD50) for MSG is 15-18 g/kg body weight in rats and mice.[20] The dose of 2 g/kg of body weight corresponds to 140 g of MSG intake in a 70 kg man. The toxic effects on central nervous system, adipose tissue and liver, reproductive organs and other systems have been shown when the MSG intake was between 3-6 mg/kg of body weight in rats and mice.[21-23] However, there is no restriction to utilize in foods and cooking as additive by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).[24] It has been decades the researches have been searching the effects of MSG on many aspects of metabolism. However, the results of studies are still controversial.[20,22]

In the present study, we aimed to define the effects of maternal MSG intake on the learning ability and memory of offspring which had no MSG supplement.

METHODOLOGY

Housing and feeding

Experiment was conducted on 30 female Spraque Dawley rats (6-8 weeks old) and on their pups (9 weeks old). Animals were obtained from the animal house of Istanbul Medipol University. Average weight of adult rats was 163.50±11.12 g. The animals were housed under controlled conditions. The room temperature was 23±2°C and air humidity 50±5%. The rhythm of light and darkness was established (light phase from 6:00 a.m. to 6:00 p.m.). The female rats were housed in standard cages. They had free access to tap water and to standard laboratory chow pellets. After the two weeks adaptation period, the animals were divided into two groups each including randomly selected rats. Control group (N=10) received standard diet all through the experimental period. The defining of the animals was succeeded via coloring their tails in different dyes during the experiment. All procedures on animals followed Guideline for work on experimental animals approved by Ethic Committee of Istanbul Medipol University.

MSG supplementation

MSG (mw 169.11 g/mol, purity 99%) under the license of Ajinomoto co.INC. (Tokyo, Japan) was bought in open market in Istanbul of Turkey. The solution of 30 g MSG/100 mL (1.77 M) was prepared in distilled water. The daily dose for MSG was based on the levels reported by previous studies.[21-23] In the present study, the MSG supplementation (4 mg/g.bw) was applied orally by gavage. The experimental group (N=20) was exposed MSG in every two days till the mating period (3 weeks). Due to their sensitivity, all animals had only standard chow and water ad *libitum* during one week of mating period. Then, MSG supplemented 20 rats were divided into two groups. Group I (N=10) had no MSG and group II (N=10) supplemented with MSG during pregnancy and lactation (6 weeks); Group I and group II have been also continued to standard feeding ad *libitum*. MSG was exposed one in every 3 days during pregnancy and lactation. The pups had no MSG supplementation but standard diet all through the experimental period.

Breeding was succeeded with the ratio of 1 male/3 females (harem) in a large cage. Each group of pregnant rats were taken off separate cages. Pregnant females were not housed with non-pregnant/non-breeding females. One week prior to birth, females were housed in a separate cage and rat chow and tap water continuously available. Females were checked daily by a veterinary for the birth of pups. A week after birth, mothers and their pups were placed in the experimental cages. All pups were lactated. At the end of lactation period randomly selected 7 female pups per group were subjected to Barnes maze test. After completed weaning term (3 weeks) the mother rats and the rest of the pups were sacrificed under 100 µL/kg of Ketarum (1.2 mL Ketasol 100, (Graeub), 0.8 mL Rompun 2% solution, (Bayer) diluted in 8 mL 0.9% NaCl, (Bichsel)).

Barnes maze test

Adult rats (11-13 weeks old, before mating) and their offspring (9 weeks old) underwent testing in the Barnes maze task to evaluate the spatial learning and memory.[17] This dry-land maze consisted of a circular platform at a height of 140 cm, with 20 holes along the perimeter. In this test, the animal was placed in the center on an open elevated platform and allowed to search for an escape box beneath one of the holes. Aversive stimuli sound (85 dB) was used to induce searching and finding the target box. Once the animal entered the target box, the stimulus was disabled.

In pre-training trial, the rat placed in the middle of the maze under a dark colored box allowing the rat to be in random orientation before each trial. After 10 seconds loud voice, the chamber was removed off and the rat was allowed to ex-

plore the maze. During the trials, rats from each group were randomly assigned to locate the target box. During acquisition and testing the number of false trials and the duration of trials were recorded. False trials were defined as nose pokes and head deflections over any hole that did not have the target box. The duration of trial was defined as the time it took to locate target box. The location of the goal hole was fixed across all trials and days.

Rats were trained for four trials per day with an inter-trial interval of at least 15 minutes till the average training duration reached less than 10 seconds. The number of trials, the number of false trials and the duration of trials were recorded for each rat. Following each trial, the entire maze was cleaned with 70% alcohol. A week after the group of rats reached less than 10 seconds, a probe trial was conducted to evaluate long-term memory retention without any training during the week before probe trial. Learning ability in the Barnes maze was assessed by the total numbers of false trials committed before entering the target box and the duration of trials. Memory was determined by the probe measurements. Experienced observers recorded the experimental data for each rat.

Statistical analysis

The quantitative variables were expressed as means ± standard deviation. The values at <10 seconds were also given as median ± IQR (interquartile range). All statistical analyses were performed using the SPSS statistical software (version 22.0). The study has a power of 80% involving 10 rats in each group with large effect size at the level of 5% error.

RESULTS

The Barnes maze task was applied to evaluate cognitive deficits in learning and memory of MSG exposed mother rats and their pups which had no MSG through the experimental period. The duration of trials and the number of false trials during training and probe trials were placed in Table 1.

Table 1. Barnes maze testing results of mother rats and their offspring

		Mother rats				Offspring					
		Control group (N=10)		MSG treated group[a] (N=20)		Control group (N=7)		Group I[b](N=7)		Group II[c](N=7)	
Day	Number of trials	Duration of trial (second)	Number of false trials	Duration of trial (second)	Number of false trials	Duration of trial (second)	Number of false trials	Duration of trial (second)	Number of false trials	Duration of trial (second)	Number of false trials
1	1	77.87 ±58.75	5.20 ±5.88	83.57 ±58.90	6.90 ±5.18	128.82 ±41.29	11.33 ±2.94	136.40 ±62.90	11.29 ±8.71	139.24 ±62.31	9.57 ±4.96
2	5	32.60 ±16.91	7.70 ±4.85	51.83 ±49.19	10.65 ±11.33	12.80 ±8.72	2.67 ±3.44	36.96 ±23.50	4.43 ±1.90	45.73 ±53.24	6.57 ±5.29
3	9	17.69 ±10.64	2.30 ±2.31	36.40 ±25.31	7.45 ±5.47	30.57 ±26.49	8.00 ±6.16	43.55 ±41.96	9.29 ±7.59	26.03 ±13.88	5.29 ±2.06
4	13	18.77 ±9.48	5.10 ±4.58	34.40 ±40.62	8.35 ±6.39	11.24 ±11.31	2.67 ±4.68	27.32 ±15.27	4.57 ±4.04	38.41 ±37.20	9.00 ±10.21
4	15	-	-	-	-	7.78 ±4.22 (6.64 ±4.04)	1.50 ±1.76 (1.00 ±1.25)	18.51 ±13.65	3.57 ±3.41	24.00 ±17.18	5.29 ±3.09
5	17	18.14 ±10.60	2.90 ±2.84	19.91 ±10.88	4.90 ±3.31	-	-	22.66 ±18.75	4.86 ±5.15	49.14 ±62.67	7.29 ±5.99
6	21	15.72 ±11.61	4.80 ±4.80	14.45 ±8.08	3.30 ±2.70	-	-	12.54 ±7.36	2.71 ±1.98	20.53 ±22.55	4.71 ±7.97
6	22	9.88 ±6.12 (7.78 ±10.70)	2.00 ±2.49 (1.00 ±2.00)	13.22 ±8.78	3.60 ±3.10	-	-	-	-	-	-
6	24	-	-	15.08 ±12.69	3.10 ±3.83	-	-	-	-	-	-
7	25	-	-	14.35 ±10.12	4.00 ±3.46	-	-	10.71 ±8.22	2.14 ±2.19	21.70 ±13.97	4.86 ±2.79
7	27	-	-	-	-	-	-	9.42 ±6.6 (7.70 ±13.27)	2.43 ±2.30 (2.00 ±2.00)	21.04 ±23.42	6.29 ±9.59
8	29	-	-	18.31 ±6.73	5.10 ±3.87	-	-	-	-	24.72 ±24.61	5.00 ±4.47
8	32	-	-	9.89 ±5.86 (7.80 ±9.73)	1.60 ±1.50 (1.00 ±2.75)	-	-	-	-	-	-
9	33	-	-	-	-	-	-	-	-	16.89 ±12.81	4.43 ±5.50
9	34	-	-	-	-	-	-	-	-	6.87 ±2.96 (8.92 ±5.55)	1.29 ±0.95 (1.00 ±1.00)
11	Probe	-	-	-	-	11.78 ±15.36	3.00 ±4.00	-	-	-	-
13	Probe	7.20 ±5.06	1.40 ±2.11	-	-	-	-	-	-	-	-
14	Probe	-	-	-	-	-	-	16.67 ±11.60	6.71 ±4.35	-	-
15	Probe	-	-	11.47 ±6.45	2.65 ±3.17	-	-	-	-	-	-
16	Probe	-	-	-	-	-	-	-	-		

[a]Mother rats supplemented MSG during 3 weeks before mating and then divided into two groups. [b]Group I: the offspring of MSG treated rats for 3 weeks before mating. [c]Group II: the offspring of MSG treated rats for 3 weeks before mating and during pregnancy and lactation. Probe was recorded after 7 days of reaching the duration of trial time <10 seconds. The duration and the number of trials where the scores reached less than 10 seconds were written bold. Data were presented as mean of the group ± standard deviation. The values at <10 seconds were also given as median ± IQR (interquartile range).

Training continued as four trials per day till the average training reached less than 10 seconds which referred the maximum learning of rats. Among four measurements the first value on each trial day was reported in the Table 1. Control group reached >10 seconds at the 22nd trial (6th day) whereas the rats administered MSG for 3 weeks before mating reached at the 32ndtrial (8th day). On days 13 (for control group) and 15 (MSG administered group) probe trials were conducted to evaluate long-term memory retention. Control mother rats arrived to target box in 7.20±5.06 seconds with 1.40±2.11errors while MSG treated mother rats arrived in 11.47±6.45 seconds with 2.65±3.17 errors. MSG treated mother rats (n=20) showed weaken long-term memory compared with control rats (n=10). Starting from the pre-training day, control rats had shorter duration of training time for each trial day.

Additional MSG administration during pregnancy and lactation to rats (group II) affected the learning ability in their offspring more than the rats had no MSG at mentioned periods (control group and group I). The numbers of trials to reach less than 10 seconds were 34 trials for group II, 27 trials for group I and 15 trials for control group.

When the duration of trials of offspring reached less than 10 seconds the numbers of false trials for control group, group I and group II were 1.50±1.76, 2.43±2.30 and 1.29±0.95 respectively. Probe trials of offspring were 11.78±15.36 seconds for control group, 16.67±11.60 seconds for group I and 11.06±5.48 seconds for group II with the number of false trials of 3.00, 6.71 and 3.71 respectively.

The number of false trials and the duration of trials were graphed on Figure 1. The MSG treated mother rats had better Barnes maze learning in comparison with the control mother rats. The similar attitude was observed for their offspring. The duration of training of pups involved multiple daily trials spread over 4 to 9 days. The longest trial days for Barnes maze learning plotted for the pups which their mothers were exposed MSG for 3 weeks before mating and additional supplementation during pregnancy and lactation (group II). The learning ability of offspring in control group was found the lowest in comparison with the pups in other groups.

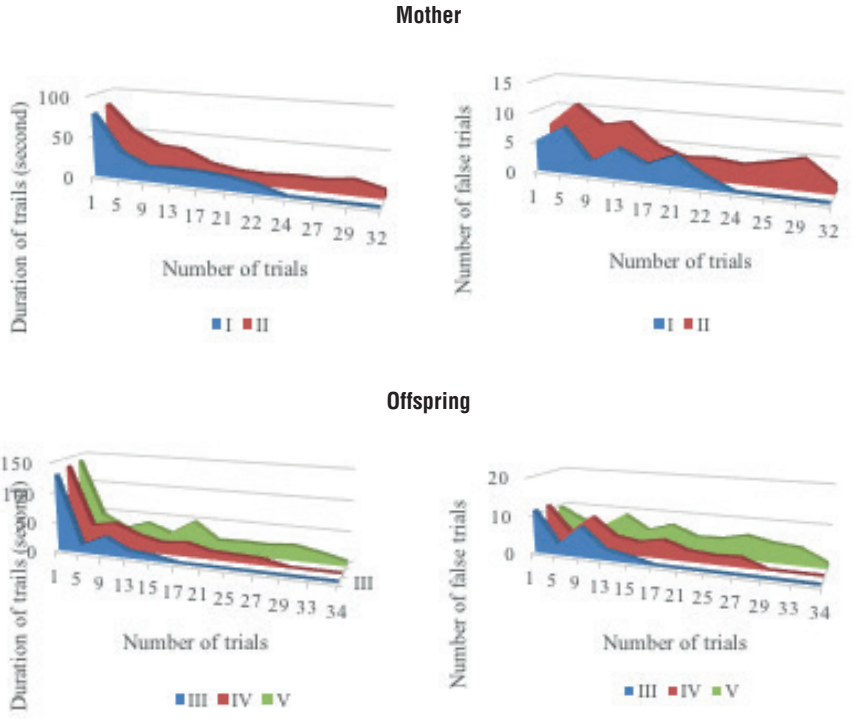

Figure 1. Number of trials vs number of false trials and duration of trials.

I: Control mother rats; II: MSG exposed mother rats for 3 weeks before mating; III: Offspring of control rats; IV: Offspring of MSG exposed mothers for 3 weeks before mating; V: Offspring of additional MSG exposed mothers during pregnancy and lactation.

DISCUSSION

Although Joint FAO/WHO Expert Committee on Food Additives (JECFA) declared no restriction to utilize MSG in foods and cooking as additive the effects of MSG intake in human and animal based studies have been evaluated for decades.[24] Moreover, toxic effects of MSG on central nervous system, adipose tissue, hepatic tissue, reproductive organs, liver and kidney functions were studied by several researchers.[22,25-27] In the studies on humans, there have been many unequal experimental conditions resulting methodological limitations, such as difficulties to apply isocaloric diet, complexity of combination of diet, etc. The animal studies are preferable because of their similarity to humans and short life cycle to study whole life span or across several generations. Tough, the doses of MSG for animals are not similar that of human intake outcome of the related studies may shed some light on the effects of dietary MSG.

In present study, MSG administered in 4 mg/g.bw of adult rats for 3 weeks decreased learning ability and memory. When MSG supplementation elongated to pregnancy and lactation periods (group II), their offspring were affected more than that of control group (no MSG intake), and group I (MSG intake for 3 weeks before mating). It should be important to notice that the pups were not given MSG during experimental period. The observed effects of MSG on the learning ability and memory of offspring may arise from the maternal MSG intake. When high quantities of MSG were administered to mammals during the neonatal period it has been shown to promote a neuroendocrine dysfunction. The maternal transportation to fetal brains and kidneys following the glutamate supplementation to pregnant mice was shown by studies with radiolabeled ^{3}H-glutamate.[28] Thus, MSG is believed to involve the glutamate-induced degeneration of certain areas of the immature neonatal brain. However, in another study MSG was given at a late stage of pregnancy. Supporting our findings, Y-maze discrimination learning of 32- and 52 days old filial mice from mothers treated with MSG was significantly less than that of the control.[29] JECFA in 2007 and 1988 defined that glutamate did not transport through fetal circulation and did not pass the placental barrier. [24,30] However in a study with pregnant rhesus monkeys, it was shown that it would be necessary a very high serum maternal concentration of glutamate, of about 2.800 μmol/L, to transfer of glutamate from the mother to the fetus to take place.[31] As a result, the JECFA allocated an "acceptable daily intake (ADI) not specified" to glutamic acid and its salts. In addition, no additional risk to infants was indicated. In a contradiction, our data indicated the slow learning ability for offspring of MSG treated rats. When we compared the learning ability and memory of pups from rats which were administered MSG before mating (group I) and plus during pregnancy and lactation (group II), the adverse effect of MSG was more in group II. The numbers of trials to drop less than 10 seconds were the highest for group II (33 trials) then for group I (27 trials) and the least for control group (15 trials) indicating the defect of maternal MSG intake on learning ability of the pups. In a same manner, the average number of false trials reached up to 1.50 (day 4) for control group, 2.43 (day 7) for group I and 4.43 (day 9) for group II. There are several studies to explore the influence of MSG directly given to adult rats or to their pups. However, our study, the first time, showed that there is an adverse effect of maternal MSG intake on learning ability of pups which did not take dietary MSG. In other words, MSG may transport from mother to fetus and cause brain damage. Leon *et al.* suggested that glutamate administered to pregnant rats modulates adenosine A1 receptor signaling pathways in both maternal and fetal brain, showing an adenosine A1 receptor down-regulation in fetal brain, and desensitization in maternal brain.[32]

MSG in high doses caused neuronal necrosis in hypothalamic arcuate nuclei in neonatal rats.[33] MSG (4 mg/g. subcutaneously on postnatal days 1, 3, 5 and 7) led to prefrontal cerebral cortex changes including fewer neurons, shorter and less ramified dendritic processes and loss of cortical cell number from postnatal day 8-14 compared to control rats.[34,35] Increased proopiomelanocortin mRNA levels and adrenocorticotropic hormone concentration in the adenopituitary have been found in neonatal MSG-treated rats compared with controls (4 mg/g per bw, administrations intraperitoneally).[36] Furthermore, it was shown that neonates treated with MSG exhibited neuronal cell death with reduction of photoreceptor and glial cells.[37-39] Indeed, there are several studies to reveal controversial results about the effects of MSG administered in late pregnancy in animals. For instance, the effect of maternal oral administration of MSG (2.5 mg/g or 4.0 mg/g bw) at 17-21 days of pregnancy on developing mouse fetal brain showed that there was no significant difference in spatial learning between the experimental animals and controls. However, subneurotoxic doses of MSG (2 mg/g for 10 days) given per orally to rat neonates decreased learning performance at the 90th post–dosing day indicating the influence of early-life MSG exposure on behavioral aberrations in adulthood.[40]

The study has several limitations. First, the mothers and pups were not paired for the comparison of learning and memory abilities. Ten mother rats for each group were included to study. However, we randomly selected seven female pups from each group of rats. So, the mother of each pup was not definite. As a result, we could not able to detect the learning and memory behaviors of the mother and its pup as a pair. Second, the duration and the number of trials were recorded for each tested animal. However, the data evaluated through the mean values and the standard deviations of the rats in each group. When the mean of the duration of trial reached less than 10 seconds, it was accepted as the exact number of trials for learning of group. The learning and memory abilities of rats were evaluated as a group but not individually.

CONCLUSION

Barnes maze learning test revealed that the duration of trials and the number of false trials increased in MSG exposed mother rats for 3 weeks before mating. The additional intake of MSG during pregnancy and lactation influenced learning ability of pups. The learning ability of offspring was measured by the number of false trials till the average training duration less than 10 seconds. The numbers of false trials increased in the order of control group (no MSG intake), group I (maternal MSG intake for 3 weeks before mating) and group II (maternal MSG intake additionally during pregnancy and lactation). In conclusion, we hypoth-

esized that maternal MSG intake of Spraque Dawley rats (4 mg/g.bw) affected learning ability of their offspring although the pups had no MSG during whole experimental period.

CONFLICT OF INTEREST

No conflict of interest was reported by the authors

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