Nigerian Journal of Pharmaceutical and Applied Science Research, 12(4):28-33; December 2023 p-ISSN: 2971-737X; e-ISSN: 2971-7388. Available at www.nijophasr.net

Hippadine from *Crinum jagus* is active against ovarian carcinoma and melanoma cancer cell lines

[1]*Comfort A. Ogah, [1]Celina O. Ogah, [1]Olusegun S. Ajala, [2]John I. Igoli, [3]Alexander I. Gray, [3]Valerie A Ferro

[1*]Department of Pharmaceutical Chemistry, University of Lagos, Yaba, Lagos, Nigeria
[2]Department of Chemistry, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria
[3]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, G4 0RE Glasgow, United Kingdom.

ABSTRACT

Background: *Crinum jagus* (Amaryllidaceae), has been used in folk medicine for the treatment of various diseases including cancer. The plant is rich in alkaloids and other phytoconstituents. This study aimed to isolate, and structurally elucidate compounds from *Crinum jagus* bulb (an Amaryllidaceae plant) and study the cytotoxic activity of hippadine on ovarian carcinoma (A2780) and melanoma cancer (A375) cell lines.

Methods: In this study, bulbs of *C. jagus* were extracted with methanol and fractionated with n-hexane, ethyl acetate, and methanol, Column chromatography was used to isolate and purify compounds while the identities of the compounds were confirmed using HRESI and 2D NMR spectroscopy. The cytotoxic activity of hippadine was evaluated on ovarian carcinoma (A2780) and melanoma cancer (A375) cell lines using a resazurin-based test (alamar blue assay).

Results: Hippadine, β-sitosterol, stigmasterol, and 4′-hydroxy-7-methoxyflavan were isolated from the ethylacetate fraction of the bulbs. The identity of these compounds was further confirmed by comparing research data with other literature reports. This is an initial report of the flavan in *Crinum jagus*. The cytotoxic studies on hippadine gave IC_{50} values of 4.23± 0.35µg/ml on A2780 cells and 4.32 ± 0.55 µg/ml on A375 cells.

Conclusion: This study has justified the use of *Crinum jagus* bulb in folkloric medicine for the treatment of cancer. The phytochemical constituents hereby reported and the cytotoxic effect of hippadine provides a basis for further investigation into other pharmacological uses of *Crinum jagus* bulb as alkaloids are known for their diverse pharmacological activities.

Keywords: Amaryllidaceae, Alkaloids, Cytotoxicity, Cancer, Fractionation

1.0 INTRODUCTION

The Amaryllidaceae family contains a group of wild and bulbous plants and are considered ornamental due to their beautiful flowers. Investigations into this plant group started because of their broad spectrum pharmacological activities which has been attributed to their alkaloid-rich constituents [1, 2, 3]. The plants have shown diverse biological activities such as; antitumoral cytotoxicity [4], reversible inhibition of acetylcholine [5], antibacterial and anti-parasitic activities [6], anti-inflammatory and antioxidant activities [4]. In 1877, the first Amaryllidaceae alkaloid, lycorine, was isolated from *Narcissus pseudonarcissus*. Several phytochemicals have been isolated from the Amaryllidaceae family [7]. As new compounds are discovered, many more biological targets are also being explored. This continues to give relevance to the Amaryllidaceae plants as potential source of future drugs. In this study, hippadine, two phytosterols and a flavan were isolated from *Crinum jagus* bulbs and the cytotoxic activity of hippadine against ovarian cancer A2780 and human melanoma A375 cell lines was also evaluated.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

NMR Spectrophotometer – 400MHz (Bruker, USA), HRESI-Mass Spectrophotometer (Thermoscientific®), Rotary Evapourator (R-3, BTECH, Switzerland), Glass columns (Pyrex). Silica gel 0.063– 0.200 nm mesh, thin layer chromatography (TLC) plates (precoated aluminum sheets with silica

* Corresponding author: Email: ogahcomfort@gmail.com; Phone: +2347038704448

2.1.2 Chromatographic and Biological Materials
Silica gel 60 F_{254} from Merck, UK, Resazurin (AlamarBlue®), Penicillin–Streptomycin. Ethyl acetate, n-hexane, methanol, Roswell Park Memorial Institute (RPMI) 1640 medium and foetal bovine serum (FBS) were purchased from Fischer, UK. Sephadex LH 20 from GE Healthcare, UK and the cell lines from the European Collection of Authenticated Cell Cultures (ECACC, UK), Forestry Research Institute of Nigeria, Ibadan, Nigeria (Plant voucher deposit site), Voucher number FHI 113600

2.2 Methods
2.2.1 Isolation of compounds
C. jagus bulbs were collected from the botanical garden of the Forestry Research Institute of Nigeria, Ibadan, Nigeria in September 2020. A voucher specimen was deposited at the Institutes' herbarium. Air-dried and powdered bulb material (800 g) was exhaustively extracted using methanol in a Soxhlet extraction set-up. The extract was filtered and concentrated to dryness using a rotary evaporator to yield 40 g of extract. The solvent free methanol extract was fractionated by trituration into n-hexane, ethyl acetate and methanol fractions. The ethyl acetate fraction (5 g) was further fractionated by chromatographic method using silica gel as stationary phase, and eluted with n-hexane:ethyl acetate mixtures gradient wise. This procedure afforded 4 main fractions from 10:0, 8:2, 5:5, 0:10 mixtures as fractions 1, 2, 3, and 4, respectively. Fraction 1 was subjected to column chromatography using silica gel and purification of its sub-fraction yielded compounds **1**, **2,** and **3**. Fraction 3 was purified using silica gel as above and subsequently with gel permeation chromatography using Sephadex LH-20 and eluted with methanol to yield compound **4**.

2.2.2 Spectroscopic analysis
The high-resolution masses of the compounds were obtained on an Ultra High-Performance Liquid Chromatography Accela system coupled to an Exactive-Orbitrap high-resolution mass spectrometer and their NMR spectra were acquired on a Bruker Avance III (400 MHz) spectrophotometer.

2.2.3 Cell culture
Human ovarian carcinoma (A2780) and melanoma (A375) cell lines were cultured in sterile T25 flasks containing RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/ streptomycin at 37°C, 5% CO_2, and 100% humidity. When the cells were approximately 70%–80% confluent, they were trypsinised from the culture flasks and 100 µL of cells were seeded in flat bottomed 96-well plates at a concentration of 5×10^4 cells/mL. The plates were incubated for a further 24 h before treatment with hippadine.

2.2.4 Alamar blue assay of hippadine on A2780 and A375 cell lines
Hippadine was evaluated for *in vitro* cytotoxicity using a Resazurin-based assay [8]. After a 24 h incubation, cells were treated with hippadine dissolved in ethanol (0.04 – 88 µg/mL concentrations) at 37 °C, 5 % CO_2 and 100% humidity for 24 h. The solvent control was 100% ethanol (100µL). Next, cells were incubated with 20 µL Alamar blue (0.1 mg/mL) and incubated for a further 24 h. Metabolically active cells were calculated as a percentage of the untreated control cells.

2.3 **Statistical Analysis**
Statistical analysis was carried out using GraphPad Prism 5.0, via two-way Analysis of Variance (ANOVA). Experiments were in triplicate and results were expressed as a mean ± SD of the readings.

3.0 RESULTS

Table 1: Percentage yield of extract, fractions and compounds

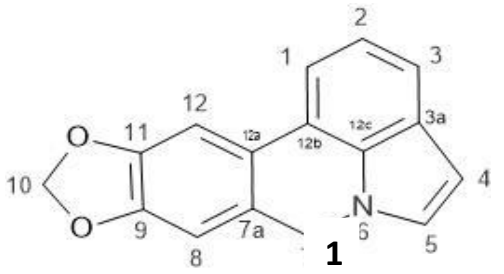
Plant name and part	*C. jagus* bulb
plant material (g)	836
crude methanol extract (%)	5.3
n-hexane fraction (%)	12.5
Ethyl acetate fraction (%)	22.7
methanol fraction (%)	61.3
Compound **1** (mg)	17.9
Compound **2** & **3** (mixture) (mg)	65.8
Compound **4** (mg)	36.1

3.1 Isolation and purification of compounds 1, 2, 3 and 4
Fraction 1 from the ethyl acetate fraction (5 g), was subjected to column chromatography and 4 fractions were obtained. The first fraction was subjected to further separation over an open column followed by TLC analysis. Purification of pooled samples on Sephadex™ LH-20 yielded 17.9 mg of compound 1 and 65.8 mg of a mixture of compounds 2 and 3. A difficulty in separating compound 2 and 3 was observed after multiple purification efforts.

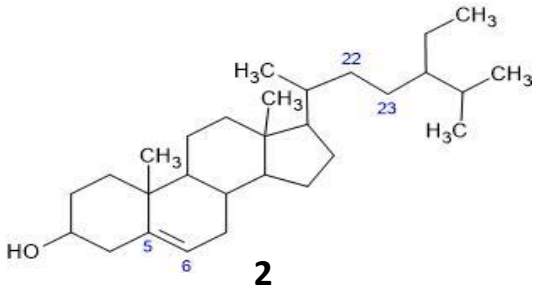
The second fraction was also separated on a column and fractions collected were purified. With the guidance of TLC analysis of fractions collected during purification, compound 4 was isolated. Its yield was 36.1 mg. The yields of the crude extracts of *C. jagus* bulbs with methanol, its fractions with n-hexane, ethyl acetate and methanol and yields of isolates are presented in Table 1.

3.2 Structural elucidation of compounds

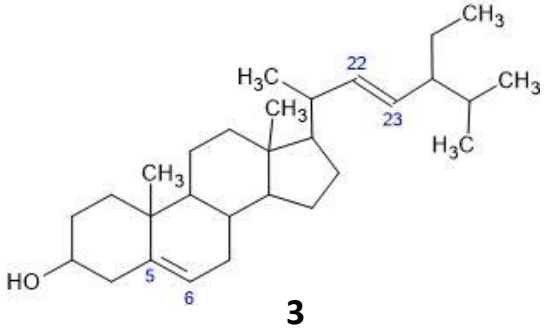
Compound **1** was isolated as an off-white colored powder with a molecular ion at m/z 264.0658 $[M^+H]^+$ (calc. 264.0661, $C_{16}H_{10}O_3N$) corresponding to the molecular formula $C_{16}H_9O_3N$. The proton NMR showed signals for three coupled aromatic protons at δ_H 7.44 ppm (t, *J* = 7.7 Hz, H-2), δ_H 7.72 ppm (dd, *J* = 7.7 Hz, H-3) and δ_H 7.87 ppm (d, *J* = 7.7 Hz, H-1)) and two other aromatic protons at 7.93 ppm (s) and 7.60 ppm (s). Also, there were two olefinic protons at 8.01 ppm (d, *J* = 3.6 Hz) and δ_H 6.87 ppm (d, *J* = 3.6 Hz) and two methylenedioxy protons at $\delta_{H\,6.14}$ ppm. The COSY spectrum showed correlations from δ_H 6.8 (H-4) to the proton at δ_H 8.0 (H-5). Using the 13C and 2D spectra, the compound was identified as hippadine and the chemical shift data were in agreement with literature reports. [9, 10, 11]. The mixture containing compounds **2** and **3** showed proton signals between δ_H 0.40 to 7.90. They were four methyl doublets at 0.93 (Me-21), 0.85 (Me-26), 0.83 (Me-29) and 0.81 (Me-27) and two singlets at 1.01 and 0.69 were attributed to Me-19 and Me-18 respectively. Two signals at 5.15 and 5.05 were assigned to the olefinic protons H-22 and H-23 of compound **3**



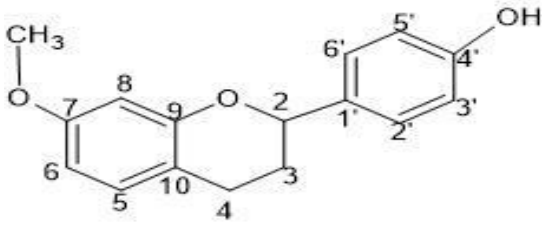
1



2



3

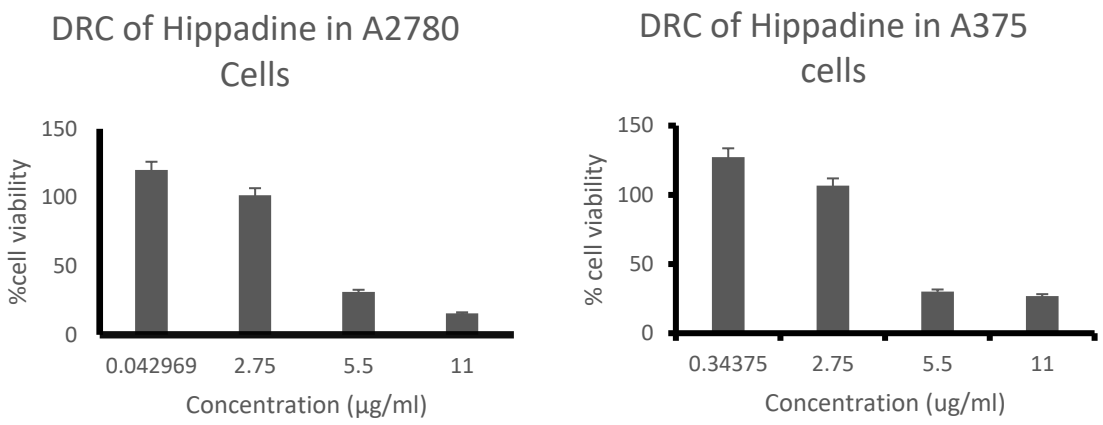


4

while the signal at 3.47 ppm was for the H-3 proton. The DEPT NMR spectrum showed signals at δc 71.9 and 138.4 ppm corresponding to the C-3 and C-22 carbons, respectively, on the sterol nucleus. Olefinic carbon signals were observed at δ 140.8 ppm and 121.8 corresponding to C-5 and C-6 respectively. Other signals at δ 138.4 and δ 129.1 were assigned to C-22 and C-23 due to the olefinic carbons of compound **3**. The rest of the carbon signals were within chemical shifts for the mixture of compounds **2** and **3** and they were identified as phytosterols β-sitosterol and stigmasterol (Fig. 3) on comparison with literature reports [12, 13]. The ^{1}H NMR of compound **4** showed aromatic proton signals at δ_H 7.21 (1H, d, *J* = 8.4 Hz), 6.36 (1H, d, *J* = 2.1 Hz, H-8), 7.23 (1H, d, *J* = 8.4 Hz, H-2′), 6.43 (1H, d, *J* = 2.1 Hz, H-6) In addition there was an oxymethine proton signal at 4.93 (1H, s, H-2), and a methoxy group at 3.65 (3H, s). The DEPT NMR spectrum of 4'-hydroxy-7-methoxyflavan showed signals at δ 24.4, 29.6, 55.1, 77.5, 101.8, 107.3, 114.3, 115.5, 128.0, 130.4, 156.1, 157.4 and 159.0. The signal at δ 24.4 ppm corresponds to position C-4 of the chromenone ring, while the signals at δ 128.0 (C-2'), 115.5 (C-5') and 128.0 (C-6') are assigned to the carbons of the phenolic ring. The signals at δ 77.5 (C-2), 130.4 (C-5), 107.3 (C-6), 159 (C-7), δ 101.8 (C-8), 156.1 (C-9) and δ 55.1 (OCH3) group are of the flavan ring. The spectral data corresponds to information reported by [14, 15] and identified as 4'-hydroxy-7-methoxy flavan.

3.3 Cytotoxicity dose-response studies on hippadine

To ascertain suitable concentrations of drug for cytotoxicity assay, dose response studies were conducted with selected concentrations of drug on cells. The results showed an IC_{50} of 4.5µg/ml for A2780 around and A375 around 5µg/ml. The dose-response data provided a guide for further studies on the cytotoxicity and IC_{50} calculations (Figure 2).



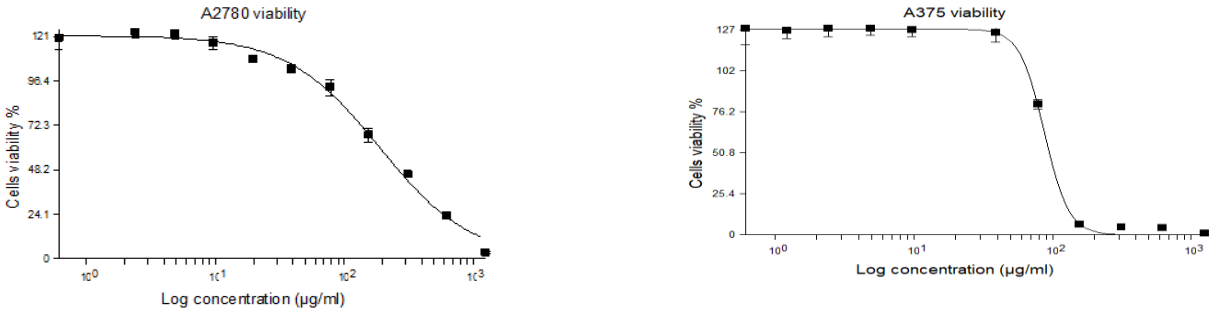
2A 2B

Figure 2 (A & B): Dose–response of hippadine on A2780 and A375 cell lines.

Cell viability was assessed using Alamar blue assay method. Each bar represents mean percentage cell viability ± SD (n=3) P<0.05

3.5 Cytotoxicity studies on hippadine against Human ovarian carcinoma (A2780) and melanoma cells (A375)

The cytotoxicity of hippadine against A2780 and A375 cell lines after 24h are shown in figure 3(A and B) and the IC_{50} of 4.32 ± 0.55 µg/ml and 4.23± 0.35µg/ml were obtained for the cell lines respectively



3A 3B

Figure 3: Percentage cell viability of hippadine on (A) A2780 and (B) A375 cell lines

Results are expressed as percentage cell viability of untreated cells. The results represent mean± SD from three independent experiments (p<0.05).

4.0 DISCUSSION

C. jagus has shown the presence of various bioactive compounds, including an alkaloid, a flavonoid, and two phytosterols. While the alkaloid and phytosterols have been previously reported from this plant [8], this is the first report of the flavonoid occurring in *Crinum jagus.* Several reports [16] stated the presence of hippadine in several Amaryllidaceae plants. With a yield of 565mg of hippadine from 10 kg of plant material (*Crinum latifolium*) [16], it indicates a high amount of hippadine among the Amaryllidaceae plants. Kaemferol, a flavonoid, has been reported from *Crinum jagus* leaves extract [17]. Flavonoids have been shown to be involved in the reduction of oxidative stress. A report on *C. jagus* shows its antioxidant activity [18]. In this study, the alkaloid isolated from this plant, hippadine, has shown promising cytotoxic activity against human ovarian (A2780) and melanoma (A375) cell lines. Previous studies on hippadine has shown cytotoxicity against several cancer cell lines, including lung, colon, and breast cancer cells, with IC50 values ranging from 0.5 to 4.7 µM [19, 20]. These studies on hippadine have demonstrated its potential as an anticancer agent. Hippadine has been reported to have activity against multidrug resistant human breast cancer cells via inhibition of P-glycoprotein expression and function [21]. The cytotoxic activity of hippadine against cancer cells suggests its potential as an anticancer agent, while not much has been

reported on the pharmacology of the flavan, the phytosterols have shown various biological activities which include; cytotoxicity, hypoglycemic, anti-inflammatory, among others [22]. These may also account for other potential health benefits attributed to *C. jagus*. Further studies are needed to investigate parameters for improved pharmacological response (such as drug delivery methods) of these compounds and their potential for use as lead drugs.

5.0 CONCLUSION

In conclusion, hippadine isolated from *C. jagus* has shown good activity against A2780 ovarian carcinoma and A375 melanoma cells. The presence of hippadine and its cytotoxic activity validates the use of the plant in traditional practice for the treatment of tumors. The use of Crinum latifolium L. (Amaryllidaceae) in Asian folk and traditional medicine in the treatment of various illnesses like rheumatism, fistula, tumors, earaches, rubefacient, tubercle and whitlow, [23], are mainly attributed to the presence of many different alkaloids which includes hippadine. Nguyen [16] reported on several hippadine-containing preparations circulating the Vietnamese market as at 2018 and went further to isolate hippadine from bulbs of Crinum latifolium L. for the standardization of such herbal preparations.

Acknowledgment

The authors wish to thank the Tertiary Education Trust Fund (TETFund), Nigeria for the funding of this research.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Contribution of the Authors

Comfort A. Ogah- Idea generation, Laboratory analysis, and manuscript development; Celina O Ogah - Idea generation, supervision, and manuscript editing; Olusegun S Ajala – Supervision; John I. Igoli - Supervision and manuscript editing; Alexander I Gray – supervision; Valerie A Ferro - supervision and manuscript editing.

6.0 REFERENCES

[1] Marco M., Roberta D.L., Allesio C. and Antonio E. Review- Advances in the Chemical and Biological Characterisation of Amaryllidaceae Alkaloids and Natural Analogues Isolated in the Last Decade. Molecules 2020; 25: 5621.

[2] Adesina, S. K., Gbolade, A. A., Sanya, O., & Omikorede, O. Alkaloid constituents of Crinum jagus. Nigerian Journal of Natural Products and Medicine 2014; 8: 26-30. https://doi.org/10.4314/njnpm.v8i1.11777

[3] Bastida, J., Lavilla, R., & Viladomat, F. Chemical and biological aspects of Amaryllidaceae alkaloids. In The Alkaloids: Chemistry and Biology 2006; 63:87-179. Elsevier. https://doi.org/10.1016/S1099-4831(06)63002-4

[4] Liu, Z.M.; Huang, X.Y.; Cui, M.R.; Zhang, X.D.; Chen, Z.; Yang, B.S.; Zhao, X.K. Amaryllidaceae alkaloids from the bulbs of Lycoris radiata with cytotoxic and anti-inflammatory activities. Fitoterapia 2015; 101: 188–193.

[5] Lahiri, D.K.; Farlow, M.R.; Greig, N.H.; Sambamurti, K. Current drug targets for Alzheimer's disease treatment. Drug Dev. Res 2002; 56: 267–281.

[6] Osorio, E.J.; Robledo, S.M.; Bastida, J. Alkaloids with antiprotozoal activity. Alkaloids Chem. Biol 2008; 66:113–190.

[7] Seydou K., Manoj K., Natacha M. and Isabel D. Biosynthesis and Biological Activities of Newly Discovered Amaryllidaceae Alkaloids- A review. Molecules 2020; 25: 4901.

[8] Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. Methods Mol Biol 2017; 1601:1-17. doi: 10.1007/978-1-4939-6960-9_1. PMID: 28470513.

[9] Kouadio A.T.G., Kabran G.R.M., Mamyrbekova-Bekro J.A., Lebrun A., Virieux D., Pirat J. and Bekro Y. Alkaloids isolated from *Crinum jagus* L. bulb (Amaryllidaceae) from Côte d'Ivoire. J Pharmacogn Phytochem 2021; 10(2):36-39. DOI: 10.22271/phyto.2021.v10.i2a.13694)

[10] Ghosal, S., Rao, P.H., Jaiswal, D.K., Kumar, Y., Frahm, A.W. Alkaloids of *Crinum pratense*. Phytochemistry 1981; 20: 2003–2007

[11] Masi, M., Cala, A., Tabanca, N., Cimmino, A., Green, I.R., Bloomquist, J.R., Van Otterlo,W.A.L., Macias, F.A., Evidente, A. Alkaloids with activity against the Zika virus vector *Aedes aegypti* (L.) —Crinsarnine and sarniensinol, two new crinine and mesembrine type alkaloids isolated from the South African plant *Nerine sarniensis*. Molecules 2016*;* 21: 1432

[12] Chaturvedula, V.S., & Prakash, I. Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of *Rubus suavissimus*. International Current Pharmaceutical Journal 2012; 1*: 239-242.*

[13] Ugochukwu C.A., Okenwa U.I., Friday C. and Juliet C.I. Isolation and Characterization of Stigmasterol and B-Sitosterol from the leaves of *Emilia coccinea* (Sims) G. Don. Communication in Physical Science 2020; 6(2):883-868.

[14] Atsushi N., Tsuruko T., Hideyuki O., Takako K., Kyoko Y., Kimihisa S. and Shigeru K. (1983). Antifeedants for the larvae of the yellow butterfly, *Eurema hecabe* in *Lycoris radiata*. Chem. Pharm. Bull 1983; 31(6): 2146-2149.

[15] Ying L., Jinhui Y., Weidong Z.L., Liming H., Jijun X., and Yulin L. (2001). Studies on Flavans. 1. Facile Synthesis of 7-Hydroxy-3′,4′-methylenedioxyflavan and 4′-Hydroxy-7-methoxyflavan by a BF3, Et2O-Mediated Pyran Cyclization. J. Nat. Prod 2001; 64:214-216

[16] Nguyen T.T.H., Huynh C.T., Phan V.H.N. and Vo T.B.H. Isolation of Hippadine from the bulbs of *Crinum latifolium* L. Amaryllidaceae. Southeast-Asian J. of Sciences 2018; 6(1):73-79

[17] Taiwe, G.S., Tchoya, T.B., Menanga, J.R., Dabole, B., & Waard, M.D. Anticonvulsant activity of an active fraction extracted from Crinum jagus L. (Amaryllidaceae), and its possible effects on fully kindled seizures, depression-like behavior and oxidative stress in experimental rodent models. J.ethnopharmacology 2016; 194: 421–433.

[18] Ghosal, S., Saini, K.S., & Razdan, S. Crinum alkaloids: their chemistry and biology, Phytochemistry 1985 24(10): 2141-2156.

[19] Zhang, Y., Zhang, Z., Xu, W., Yu, L., & Liu, Y. Preparation and characterization of a hippadine-loaded poly(lactic-co-glycolic acid) sustained-release formulation. Journal of Drug Delivery Science and Technology 2015; 30: 365-372. https://doi.org/10.1016/j.jddst.2015.09.003

[20] Liu, Y., Wang, Z., Zhang, X., Yu, L., & Zhang, Z. Solubility and stability of hippadine hydrochloride in aqueous solution. *Journal of Solution Chemistry* 2016; 45(12): 1787-1799. https://doi.org/10.1007/s10953-016-0536-7

[21] Wang, H., Zhang, Y., Wu, Q., Yu, L., & Liu, Y. Hippadine overcomes multidrug resistance in human breast cancer cells via inhibition of P-glycoprotein expression and function. Anti-Cancer Agents in Medicinal Chemistry 2019; 19(6): 801-808. https://doi.org/10.2174/1871520619666181227125432

[22] Navpreet K., Jasmine C., Akash J. and Lalit K. Stigmasterol: a comprehensive review. International Journal of Pharmaceutical Sciences and Research 2011; 2(9): 2259-2265

[23] Ghosal, S., Saini, K.S., & Razdan, S. (1985). Crinum alkaloids: their chemistry and biology, Phytochemistry 1985; 24(10): 2141-2156