CHAPTER 7

ISOLATION OF BIOACTIVE COMPOUNDS FROM *HIPPOBROMUS PAUCIFLORUS*

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Introduction

Bacterial and viral eye infections constitute a serious problem, especially in developing countries. Herpes simplex virus type 1 (HSV-1), *Staphylococcus aureus* and *Bacillus cereus* are the most frequent pathogens of eye infections (Liesegang, 2001; Hirotoshi *et al.*, 2006) Several antiviral and antibiotics drugs are available nowadays, but their use is limited by a number of factors, such as high cost, low potency, poor solubility and emergence of resistance strains. Therefore, the search for new and/or more effective antimicrobial agents continues (Penna *et al.,* 2001; Quiroga *et al*., 2001).

The purpose of this study was to isolate and identify bioactive compounds from *H. pauciflorus* through bioactivity guided fractionation of its extract, with the view to validate its usage against eye infection causing organisms.

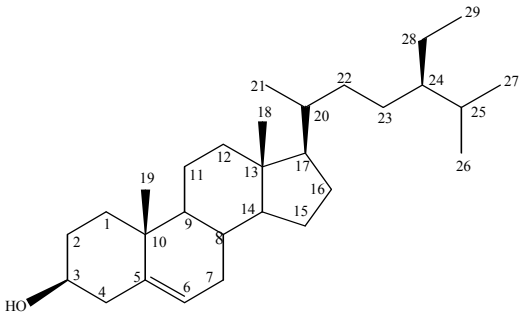
Materials and methods

Extraction and bioassay guided isolation of compounds

Dried, ground plant material (800 g) was extracted with methanol for 24 hr at room temperature three times, with gentle and continuous shaking on an orbital shaker (Stuart Scientific Orbital Shaker, UK). The extract was filtered and evaporated under reduced pressure to give 72.1 g. It was partitioned between n-hexane and water. The aqueous part was further partitioned between ethyl acetate, n-butanol and water. Each fraction was tested for antibacterial activity, using the bioautographic assay method of Slusarenko *et al*., (1989). Nutrient broth inoculated with *Bacillus subtilis* was sprayed on a developed TLC plate and incubated for 24 h at 37 °C. Bacterial growth was detected by spraying the plates with 0.2 mg/ml *p*-iodonitrotetrazolium (INT) solution and incubated at 37 °C for 30 min. The

inhibition of bacterial growth by the compounds on the TLC plate was observed as white spots against the deep red background. The ethyl acetate and n-hexane fractions showed strong antibacterial activity in the bioautographic assay.

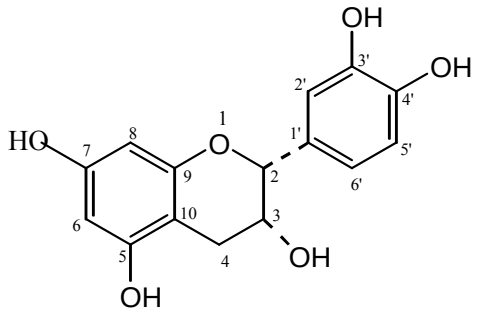
The ethyl acetate extract (18.8 g) was fractionated by vacuum liquid chromatography (VLC) over silica gel using a gradient mixture of n-hexane and acetone of increasing polarity (0-100% Acetone). A total of 33 fractions (250 ml each) were collected. The fractions were monitored by TLC and similar fractions were combined to give 13 fractions (HEVF 1-HEVF- 13). Fraction HEVF 6 (5.8 g), which was eluted with 40-60% n-hexane in acetone, was further chromatographed over silica gel column using $CHCl_3$, mixture of MeOH: $CHCl_3$ of increasing polarity to give five subfractions (HEVF 6-1 to HEVF 6-5). The subfraction HEVF 6-1 and HEVF 6-4 showed antibacterial activity. The subfraction HEVF 6-1 (1.4 g) was further fractionated by column chromatography over silica gel using $CHCl_3$: MeOH (98:5 v/v) as an eluent and a total of 3 fractions were collected. Fraction 2 was further subjected to silica gel column chromatography using $CHCl_3$: MeOH (98:2 v/v) as an eluent to yield compound **1**(β-sitosterol, 92 mg).



β-sitosterol (**1**)

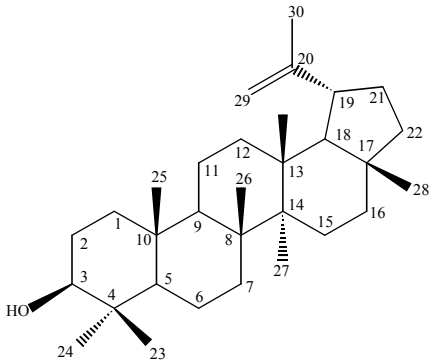
The subfraction HEVF6-4 (1.2 g) was fractionated over silica gel column using $CHCl_3$: MeOH as an eluent to give three fractions. Fraction 2 was further chromatographed by

repeated column chromatography using $CHCl_3$: MeOH (94:6 v/v) to give compound **2** (epicatechin, 10.3 mg).



epicatechin (**2**)

The n- hexane extract (23.3 g) was fractionated by VLC over silica gel using mixture of n-hexane and $CHCl_3$ of increasing polarity (0-100% $CHCl_3$) and a total of 17 fractions (250 ml each) were obtained. All the fractions were monitored by TLC and similar fractions mixed together to give four fractions (HHVF 1- 4). The fraction HHVF 3 (4.3 g), which showed antibacterial activity, was further subjected to column chromatography over silica gel using $CHCl_3$, mixture of $CHCl_3$: MeOH to give three sub-fractions (HHVF 3- 1 to HHVF 3-3). Sub fraction HHVF 3-2 (0.91g) was purified on repeated silica gel column chromatography using 2% MeOH in $CHCl_3$ to give compound **3** (lupeol, 15mg)



lupeol (**3**)

Results and Discussion

Antibacterial bioautographic assay -guided phytochemical investigation on the ethyl acetate and hexane extracts led to the isolation of three known compounds. The structures of the three compounds were identified by their spectroscopic data (UV, IR, ^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC) measurement and by comparison with published values.

Compound 1 (β-sitosterol): **^{1}H NMR** ($CDCl_3$) δ_H 5.13 (s, H-6), 3.54 (m, H-3), 0.91 (s, CH_3-19), 0.88 (s, CH_3- 18), 1.01 (d, *J*=6.3 Hz, CH_3- 21), 0.78 (d, *J*= 6.6 Hz, CH_3-26), 0.81 (d, *J*=7.2 Hz, CH_3- 27), 0.85 (t, *J*=7.8, 15.78 Hz, CH_3-29).

^{13}C NMR ($CDCl_3$, δ_c in ppm): 38.7 (C-1), 29.9 (C-2), 71.82 (C-3), 43.8 (C-4), 142.0 (C-5), 121.71 (C-6), 33.01 C-7), 31.95 (C-8), 50.17 (C-9), 37.1 (C-10), 21.5 (C-11), 40.80 (C-12), 43.0 (C-13), 56.07 (C-14), 25.8 (C-15), 29.7 (C-16), 56.90 (C-17), 12.24 (C-18), 19.44 (C-19), 36.19 (C-20), 19.82 (C-21), 39.3 (C-22), 29.5 (C-23), 29.2 (C-24), 23.11 (C-25), 18.03 (C-26), 21.27 (C-27), 23.7 (C-28), 12.1 (29).

Compound 2 (epicatechin)**: ^{1}H- NMR** (400 MHz, CD_3OD) δ 4.18 (brs, H-2), 3.31 (s, H-3), 2.86 (dd, *J*=4.4, 16.8, H-4), 2.72 (bd, H-4), 5.94 (s, H-6), 5.94 (brs, H-8), 6.98 (s, H-2'), 6.80 (d, J= 8.4 Hz, H-5') and 6.77 (d, J= 8.4 Hz, H-6').

^{13}C- NMR (400 MHz, CD_3OD) δ 79.78 (C-2), 67.39 (C-3), 29.15 (C-4), 157.48 (C-5), 96.50 (C-6), 157.82 (C-7), 95.96 (C-8), 157.25 (C-9), 100.17 (C-10), 132.20 (C-1'), 115.53 (C-2'), 145.82 (C-3'), 145.67 (C-4'), 116.00 (C-5') and 119.45 (C-6').

Compound 3 (lupeol): **^{1}H NMR** ($CDCl_3$) δ_H 4.67 (s, H-29), 4.55 (s, H-29), 3.77 (m, H-3), 2.35 (m, H-19), 1.28 (3H, s, H-30), 1.02 (3H, s, 24), 0.98 (3H, s, 23), 0.84 (3H, s, 26), 0.77 (3H, s, 27), 0.68 (3H, s, 25).

^{13}C NMR (CD_3OD + $CDCl_3$, δ_c in ppm): 38.78 (C-1), 26.00 (C-2), 78.99 (C-3), 37.19 (C-4), 55.35 (C-5), 18.02 (C-6), 34.33 (C-7), 40.87 (C-8), 50.48 (C-9), 38.10 (C-10), 20.97 (C-11), 25.19 (C-12), 38.64 (C-13), 42.85 (C-14), 26.19 (C-15), 29.71 (C-16), 47.99 (C-17), 48.35 (C-18), 47.67 (C-19), 150.88 (C-20), 29.89 (C-21), 33.35 (C-22), 27.48 (C-23), 15.38 (C-24), 16.01 (C-25), 15.60 (C-26), 14.57 (C-27), 59.91 (C-28), 109.34 (C-29), 19.33 (C-30).

Compound **1** was isolated from the ethyl acetate fraction by repeated column chromatography as a white amorphous powder and identified as β-sitosterol. By analysing the physical data, IR, EIMS, ^{1}H NMR of compound **1** and a comparative study of NMR data with the literature values of the similar compounds (Rubinstein *et al*., 1976; Sakakibara *et al*., 1983), the structure of compound **1** was determined as β-sitosterol.

Antimicrobial, anti-inflammatory, analgesic and anti-pyretic activity of β-sitosterol have been reported previously (Gupta *et al*., 1980; Salvador *et al*., 2004). It inhibited neutrophil migration into the inflamed tissue, as in acute topic inflammation it showed decreased myeloperoxidase activity (Gómez *et al*.1999). Also, β-sitosterol has been reported to have quite strong antioxidant activity (Weng and Wang, 2000).

Compound **2** was isolated from the ethyl acetate extract as brownish amorphous powder and was identified as epicatechin. The ^{1}H NMR spectrum of compound **2** showed a number of characteristic signals of epicatechin. Two signals at δ_H 5.96 (brs) and 5.94 (brs) were due to two phenyl protons situated at 1,3 position to each other on ring A. Two signals at δ_H 6.80 (d, *J*=8.4 Hz) and 6.78 (d, *J*=8.4 Hz) were due to two phenyl protons situated at ortho position and a signal at 6.98 (s) was due to one phenyl proton at para position of ring C. A singlet at δ_H 3.31 was due to methine proton (H-3) having an adjacent –OH group and

situated between methylene and a methine carbon. A singlet at δ_H 4.18 (s) for a methine proton attached with an oxygen atom and CHOH group. Two doublet of doublets resonating at δ_H 2.86 (dd, *J*=4.4, 16.8 Hz) and 2.72 (brd) were due to two methylene protons adjacent to a methine carbon.

The ^{13}C NMR data of compound **2** showed 15 carbons. The DEPT spectra revealed the presence of one methylene, seven methine and seven quaternary carbons. All connectivities between proton and carbon were determined from the COSY and HMQC experiment. The HMBC experiment of compound **2** showed strong cross peak between H-2 (δ_H 4.18) with C-3 (δ_C 67.3), C-2′ (δ_C 132.0), C-6′ (δ_C 119.4) and C-9 (δ_C 157.8).

By studying the above ^1H, ^{13}C NMR, COSY, HMQC, HMBC and comparison of the reported published spectral data (Agarwal, 2003), it was concluded that compound **2** was epicatechin and is a monomeric member of the polyphenols. It has been reported by Masika *et al*. (2004) to possess antibacterial activity against the Gram-negative bacteria, *E. coli* and *P. aeruginosa*, compared to the Gram-positive bacteria (*B. subtilis* and *S. aureus*). Epicatechin was also reported to possess antiviral activity against the herpes simplex virus 1 (Esquenazi *et al*. 2002). It has protective effects against neuronal cell death induced by oxidative stress (Abd El Mohsen *et al*., 2002). This is the main component of green tea, red grapes and apples (Ale-Agha *et al*., 2002; Xie *et al*., 2003).

Compound **3** was isolated from the hexane soluble fraction by column chromatography and was identified as lupeol. The ^1H NMR of compound **3** had shown two characteristic two olefinic signals of lupeol. These two olefinic protons appeared as a singlet at δ 4.67 and 4.55. The ^{13}C NMR of compound **3** showed thirty carbons in the molecule. The DEPT spectrum showed that it contained seven methyl, eleven methylene, six methine and six quaternary carbons. Two signals at δ_C 109.34 and 150.88 ppm were due to two olefinic

carbons of C-20 and C-29, respectively, of which the deshielded signal at δ_C 150.12 ppm was assigned for quarternary olefinic carbon C-20. The other deshielded signal at δ_C 78.99 was due to C-3 with a hydroxyl group attached to it. Comparing its UV, IR, ^{1}H- and ^{13}C NMR spectral data with the literature values of reported compounds (Mahato *et al*., 1994; Marina *et al*., 1997; Rosenel *et al*., 1998), the structure of compound **3** was elucidated as 20 (29) lupen-3β-ol (lupeol).

Diverse range of biological activities triggered by lupeol have been reported including, the stimulation of programmed cell death in human leukemia cell line (HL-60) (Aratanechemuge *et a*l., 2004). Lupeol is known to show anti-inflammatory and antiarthritic activities (Agarwal, 2003). It inhibits the growth of highly aggressive human metastatic melanoma cells (Saleem *et al*., 2008), has antiangiogenic activity (You *et al*., 2003), antioxaluric and anticalciuric activities (Anand *et al*., 1995). Lupeol derivatives have also been reported to have antimalarial activity (Fotie *et al*., 2006; Kumar *et al*., 2008). Lupeol is futher reported to possess antimicrobial activity (Ragasa *et al*., 2005).

References

Abd El Mohsen, M.M., Kuhnle, G., Rechner, A.R., Schroter, H., Rose, S., Jenner, P., Rice-Evans, C.A., 2002. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radical Biolology & Medicine 33, 1693-1702.

Agarwal, R.B., Rangari, V.D., 2003. Antiinflammatory and antiarthritic activities of lupeol and 19a-H lupeol isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* roots. Indian Journal of Pharmacology 35, 384-387.

Ale-Agha, N., Stahl, W., Sies, H., 2002. Epicatechin effects in rat liver epithelial cells: Stimulation of gap junctional communication and counteraction of its loss due to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. Biochemical Pharmacology 66, 2145-2149.

Anand, R., Patnaik, G.K., Kamal, R., Bhaduri, A.P., 1995. Antioxaluric and anticalciuric activity of lupeol derivatives. Indian Journal of Pharmacology 27, 265-268.

Aratanechemuge, Y., Hibasami, H., Sanpin, K., Katsuzaki, H., Imai, K., Komiya, T., 2004. Induction of apoptosis by Lupeol isolated from mokumen (*Gossampinus malabarica* L. Merr) in human promyelotic leukemia HL-60 cells. Oncology Reports 11, 289-292.

Esquenazi, D., Wigg, M.D., Miranda, M.M.F.S., Rodrigues, H.M., Tostes, J.B.F., Rozental, S., Da Silva, A.J.R., Alviano, C.S., 2002. Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. Research In Microbiology 153, 647-652.

Fotie, J., Bohle, D.S., Leimanis, M.L., Georges, E., Rukunga, G., Nkengfack, A.E., 2006. Lupeol longchain fatty acid esters with antimalarial activity from *Holarrhena floribunda*. Journal of Natural Products 69, 62-67.

Gomez, M., Saenz, M., Garcia, M., Fernandez, M., 1999. Study of topical anti-inflammatory activity of *Achillea ageratum* on chronic and acute inflammation models. Zeitschrift fur Naturforschung 54, 937-941.

Gupta, M.B., Nath, R., Srivastava, N., Shanker, K., Kishor, K., Bhargava, K.P., 1980. Anti-inflammatory and antipyretic activities of beta-sitosterol. Planta Medica 39 (2), 157-163.

Hirotoshi, I.B., Takashi, S., Yoshiaki, K., Kiyofumi, O.,Yasushi, I.,Wei, Z., Mohammad, M.S., Yoshihiro, K.F., Yuichi, O., Takayuki, E., 2006. Emerging multiple mutations and high level fluoroquinolone resistance in methicillin-resistant *Staphylococcus aureus* isolated from ocular infections. Journal of Diagnostic Microbiology and Infectious Diseases 56, 297-303.

Kumar, S., Misra, N., Raj, K., Srivastava, K., Puri, S.K., 2008. Novel class of hybrid natural products derived from lupeol as antimalarial agents. Natural Products Research 22, 305-319.

Liesegang, T.J., 2001. Herpes simplex virus epidemiology and ocular importance. Cornea 20, 1-13.

Mahato, S. B., Kundu, A. P., 1994. 13C NMR Spectra of pentacyclic triterpenoids- a compilation and some salient features. Phytochemistry 37, 1517-1575.

Marina, A., Andrew, H., David, T., 1997. Triterpene Esters from Australian *Acacia.* Journal of Natural Products, 60, 1150.

Masika, P.J., Sultana, N., Afolayan, A.J., 2004. Antibacterial Activity of Two Flavonoids Isolated from *Schotia latifolia.* Pharmaceutical Biology 42, 105-108.

Penna, C., Marino, S., Vivot, E., Cranes, M.C., Munoz, J.D., Cruanes, J., Ferraro, G., Gutking, G., Martino, V., 2001. Antimicrobial activity of Argentine plants used in the treatment of infectious diseases. Isolation of active compounds from *Sebastiania brasiliensis*. Journal of Ethnopharmacology 77, 37-40.

Quiroga, E.N., Sampietro, A.R., Vattuone, M.A., 2001. Screening antifungal activities of selected medicinal plants. Journal of Ethnopharmacology 74, 89-96.

Ragasa, C.Y., de Luna, R.D., Hofilena, J.G., 2005. Antimicrobial terpenoids from *Pterocarpus indicus*. Natural Product Research 19, 305-309.

Rosenel, L., Brum, N.K., Honda, S.C., Hess, A.J., Cavaleheiro, S.C., Monache, F.D., 1998. Phytochemistry, 49, 1127.

Rubinstein, I., Goad, L.J., Clague, A.D.H., 1976. The 200 MHz spectra of phytosterols. Phytochemistry 15, 195-200.

Sakakibara, S., Shiomi, K., Kobayashi, S., Ikeda, T., Inai, S., Kagamiyama, H.,1983. A convenient and sensitive method for the determination of serum aspartate aminotransferase isozymes after electrophoresis. Clinica Chimaca Acta 133, 119-123.

Saleem, M., Maddodi, N., Zaid, M. A., Khan, N., Hafeez, B.B., Asim, M., Suh, Y., Yun, J.M., Setaluri, V., Mukhtar, H., 2008. Lupeol Inhibits Growth of Highly Aggressive Human Metastatic Melanoma Cells *In vitro* and *In vivo* by Inducing Apoptosis. Clinical Cancer Research 14, 2119-2127.

Salvador, M.J., Zucchi, O.L.A.D., Candido, R.C., Ito, I.Y., Dias, D.A., 2004. In vitro antimicrobial activity of crude extracts and isolated constituents of *Alternanthera maritime*. Pharmaceutical Biology 42, 138-148.

Slusarenko, A.J., Longland, A.C., Whitehead, I.M., 1989. A convenient, sensitive and rapid assay for antibacterial activity of phytoalexins. Botanica Helvetica 99, 203-207.

Weng, X.C., Wang, W., 2000. Antioxidant activity of compounds isolated from *Salvia plebeian*. Food Chemistry 71, 489-493.

Xie, D., Sharma, S.B., Paiva, N.L., Ferreira, D., Dixon, R.A., 2003. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosythesis. Science 299, 396-399.

You, Y.J., Nam, N.H., Kim, Y., Bae, K.H., Ahn, B.Z., 2003. Antiangiogenic activity of lupeol from *Bombax ceiba*. Phytotherapy Research 17, 341-344.

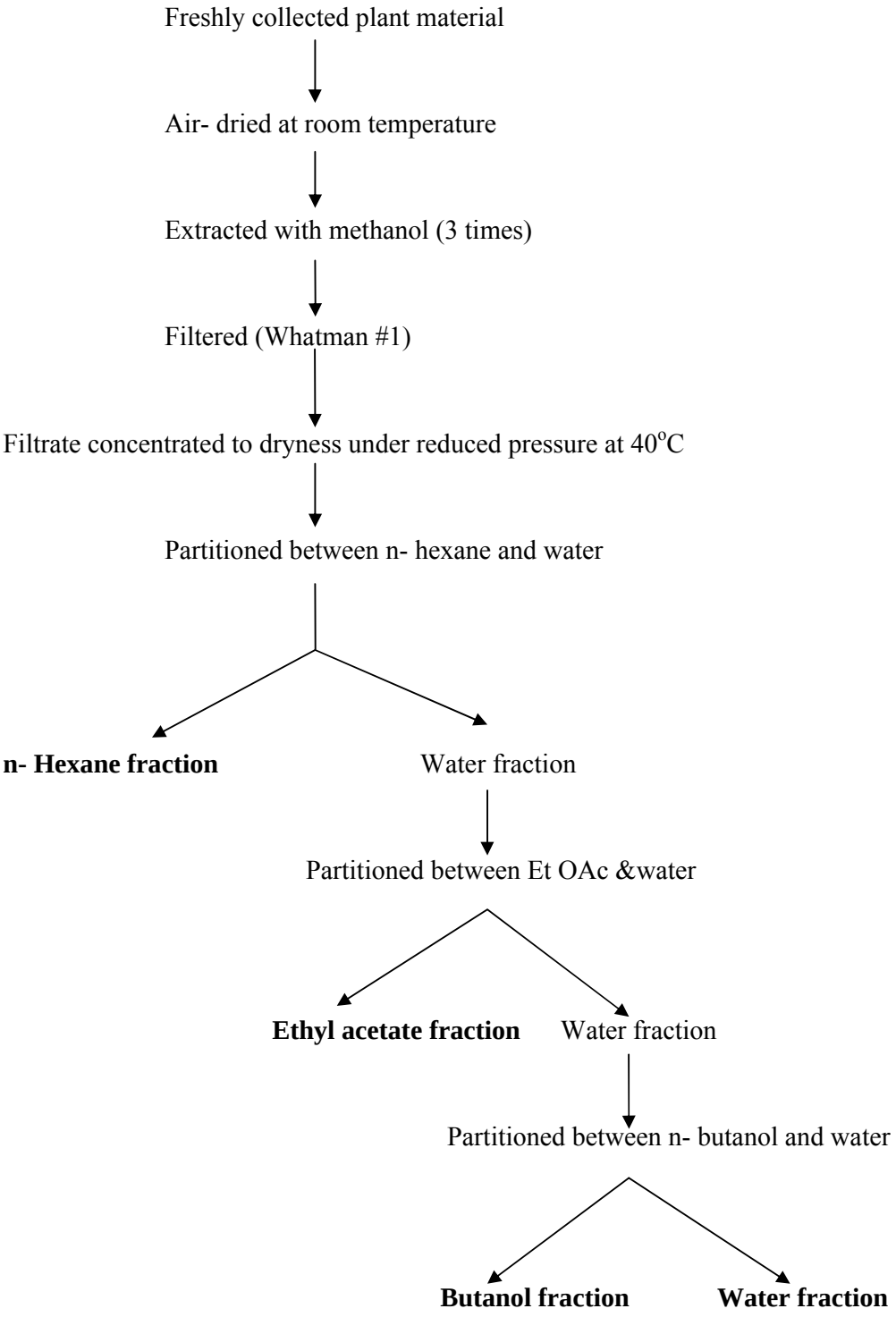


Diagram 1: Extraction and partitioning method employed for isolation of compounds from *Hippobromus pauciflorus*

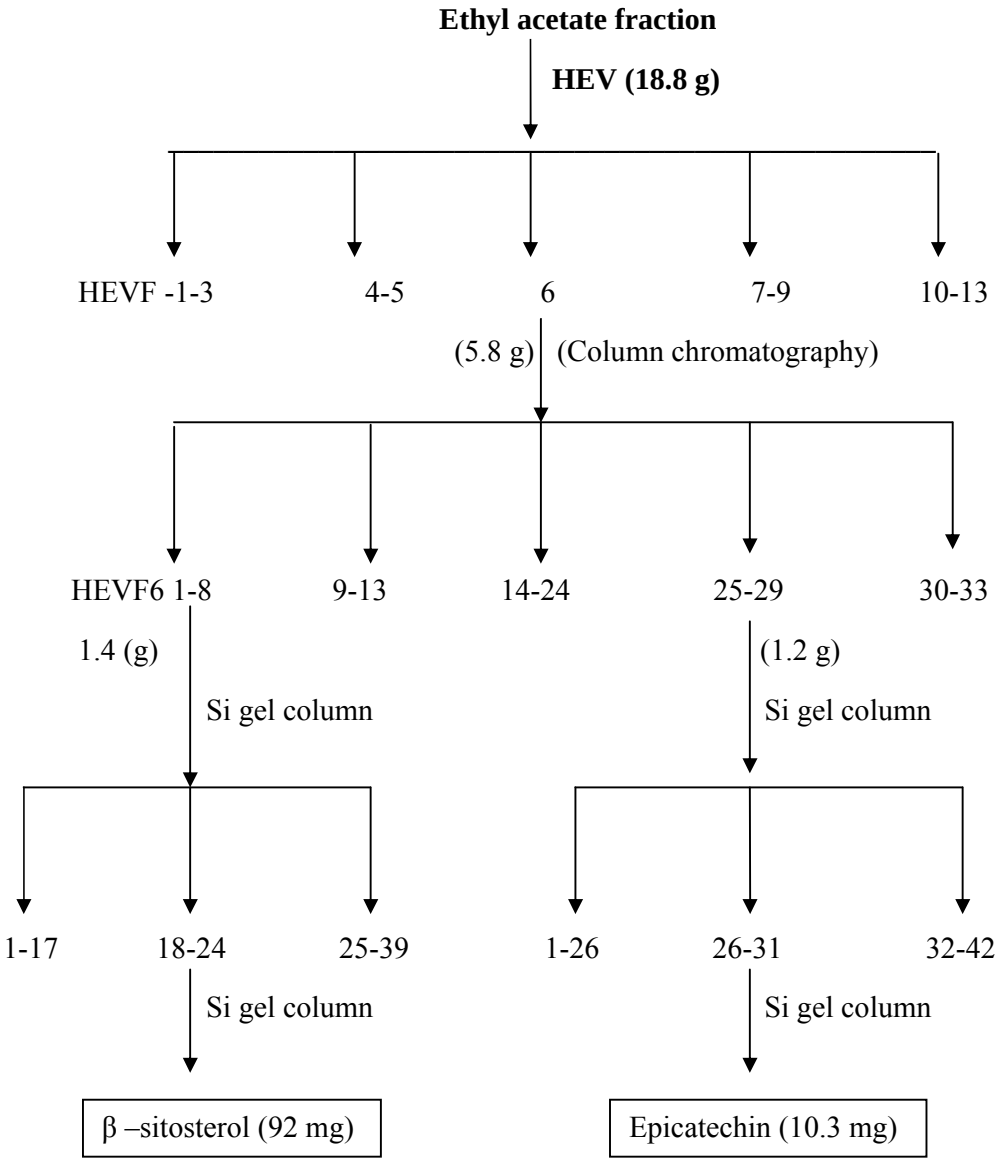


Diagram 2: Isolation of β-sitosterol and epicatechin from the ethyl acetate fraction of the leaves of *H. pauciflorus*

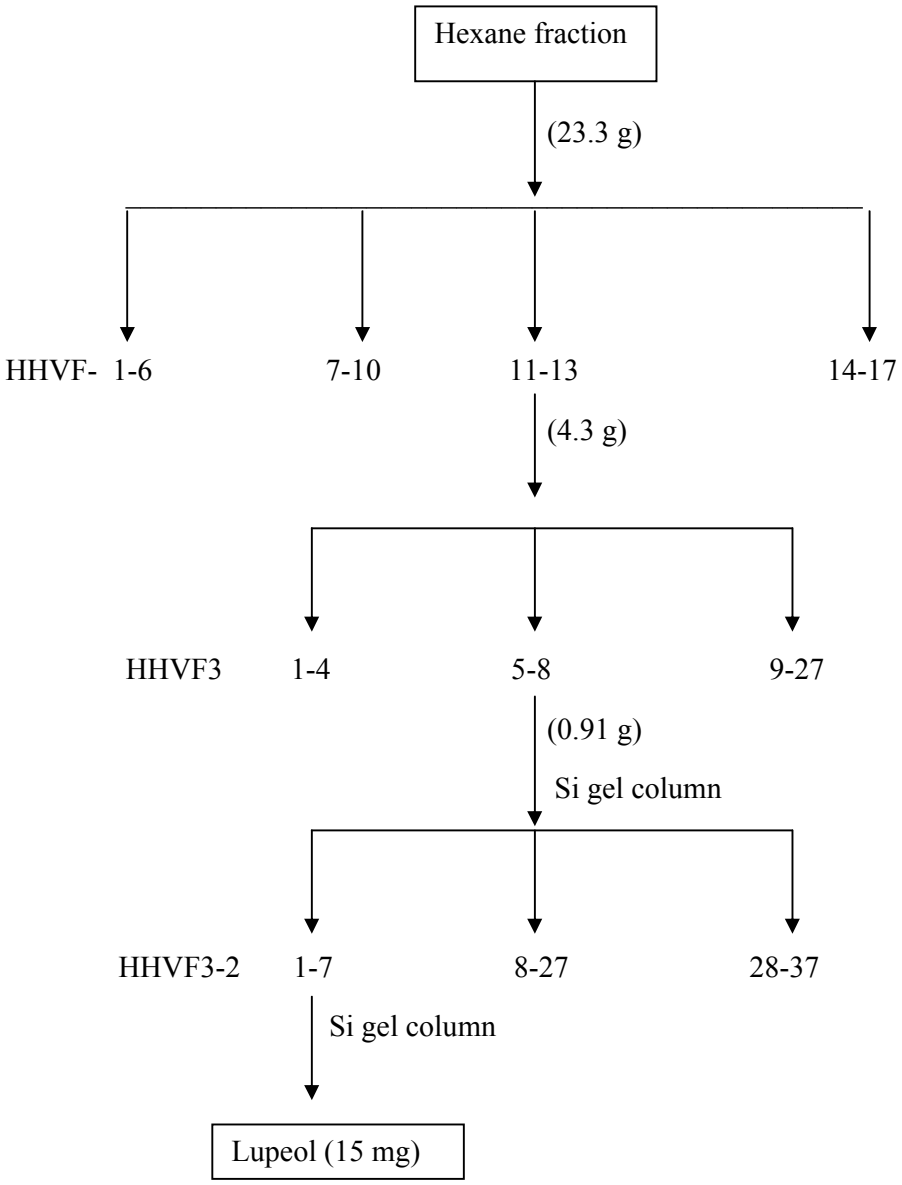


Diagram 3: Isolation of lupeol from the n-hexane fraction of the leaves of *H. pauciflorus*