EXCELLENT PUBLISHERS

International Journal of Current Research in Biosciences and Plant Biology

ISSN: 2349-8080 Volume 2 Number 5 (May-2015) pp. 124-129

www.ijcrbp.com

Original Research Article

Phytochemical analysis and isolation of Stigmasterol and β-Sitosterol from the Rhizomes of *Alpinia allughas* Rosc.

Sonali Sethi, Om Prakash* and A.K. Pant

Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar, U.S. Nagar-263 145, Uttarakhand, India

**Corresponding author.*

Abstract

Alpinia allughas Rosc. is a Zingiberaceous herb. The extract of dried rhizomes of *A. allughas* was partitioned with different solvent system by increasing their polarities (petroleum ether, hexane, dichloromethane and methanol). All these extracts were subjected to thin layer chromatography (TLC) phytochemical screening and revealed to the presence of steroids, terpenoids, alkaloids and phenolics. The total flavonoid content of the extracts was determined quantitatively and varied from 20.12±0.222 to 41.53±0.769 mg/g CNE (catechin equivalents). However the orthodihydric phenol content found in various extracts ranged from 32.00±1.802 to 54.00±0.5 mg/g CLE (catechol equivalents) quantitatively. The compounds were fractionated and isolated from petroleum ether partitionates by using column chromatography and thin layer chromatographic technique. The structure of the isolated compounds was characterized by various spectroscopic techniques such as I.R, ^{1}HNMR and ^{13}CNMR. The compounds isolated were γ-sitosterol and stigmasterol. The compounds are reported for the first time from this plant.

Keywords

Alpinia allughas
γ-Sitosterol
Phytochemical
Rhizome
Stigmasterol

Introduction

In present scenario due to the tremendous increase in the rate of infection by antibiotic-resistance microorganisms, enormous problem occurs in the treatment of infectious diseases (Sethi et al., 2013). The use of synthetic drug is prohibited due to several side effects. Thus there is a divergence from use of synthetic to natural drugs (Daayf et al., 1995). Medicinal plants are used in herbalism and are considered as rich resources of ingredients (Aguin et al., 2006). Secondary metabolites isolated from the plants are found to possess pharmacological effects on animals as well as animal system (Jaracz et al., 2004). Digoxin, morphine, taxol, quercetin, reserpine etc are some of the bioactive compounds isolated from the plants which possess nutritional value as well as the pharmacological potency (Ghani, 2003). Since plants are the rich source of the chemical constituent so there is an urgent need to explore and isolate the bioactive

components which possess the therapeutic potential to treat various diseases and ailments (Jaracz et al., 2004).

Zingiberaceae (spice family) plants are important medicinal herbs in traditional system of medicine. India is very rich in the diversity of Zingiberaceous plants (Karthikeyan, 2000). Uttarakhand region is a biggest repository of these plants (Prakash and Mehrotra, 1995). *Alpinia allughas* Rosc. (Zingiberaceae) is a perennial herb with tuberous roots, sessile leaves and pink flowers (Kirtikar and Basu, 1987). The rhizomes of *Alpinia* are well known for the treatment of inflammations, stomatopathy, pharyngopathy, hiccough, dyspepsia, tubercular glands and fevers (Warrier et al., 1993-1995). The rhizome improves digestion and voice (Chunekar, 1982). To the best of our knowledge there exist no reports on the isolation of chemical constituents from the rhizomes of *A. allughas*. In the present study, we report the isolation of the compounds from the petroleum ether extract of *A. allughas* by chromatographic and visual analysis (TLC) .The presence of steroids, terpenoids, phenols, alkaloids was determined in all the extracts. Also flavonoids and orthyodihydroxyphenol content was determined quantitatively in these extracts.

Materials and methods

Collection of plant material

Fresh rhizomes of *A. allughas* were collected from Tarai region of Kumaun hills in India. The identity of the plant was confirmed by Sumer Chandra, Systematic Botany Division of Forest Research Institute, Dehradun, where herbarium specimen nos.: 9747 and 72265 dated 12 January 2004 has been deposited.

Preparation of the extracts

The rhizomes of *A. allughas* were cut into small pieces and shade dried at room temperature. The material was then ground to fine powder. About 1.5kg of the material was extracted by successive soaking for 7 days each in different solvents of varying polarity like petroleum ether, hexane, dichloromethane and methanol. The solvent extracts were filtered using muslin cloths and concentrated using a rotary evaporator. Yields of different extracts *viz*., *Alpinia allughas*, rhizome petroleum ether extract (AARPE), *A. allughas*, rhizome hexane extract (AARHE), *A. allughas*, rhizome dichloromethane extract (AARDE) and *A. allughas*, rhizome methanol extract (AARME) were observed to be 0.83%, 0.77%, 0.73% and 0.67% respectively. The extracts were stored at 4°C for further analysis and biological activity determinations.

Phytochemical screening

To determine the class of metabolites present, various extracts of *A. allughas viz.,* AARPE, AARHE, AARDE and AARME were spotted on TLC silica gel (Merck). The TLC plates were then developed in DCM: MeOH (8:2) solvent system. Initially, the spots corresponding for a particular metabolite were visualized on the TLC plates under UV. To determine the different classes of compounds present in the different extracts, different spray reagents were also used on the TLC plates. For a general profiling of the secondary metabolites present, Vanillin-H_2SO_4 reagent was used. It can establish the presence of triterpenes, sterols and steroids. Dragendorff's reagent was used to detect the presence of alkaloids while phenolic compounds were monitored using $FeCl_3$-K_3 Fe $(CN)_6$ reagent. The TLC profiles of each extract were then assessed and compared (Bungihan and Matias, 2013).

Estimation of flavonoids

1ml of plant extract was mixed with 1.25 ml of distilled water and 75µl of 5% sodium nitrite solution. The solutions were incubated for 5 min. and then 150µl of 10% aluminium chloride solution was added. After 6 min. 500µl of 1 M sodium hydroxide and 275 µl of distilled water was added, after proper mixing of the solution the intensity of pink colour was obtained at 510 nm. The standard curve was established using various concentrations of catechin. The flavonoid content was expressed as catechin equivalents (CNE) in mg/g of dry material (Choi et al., 2006).

Estimation of ortho-dihydric phenols

One ml of the extract solution was taken and mixed with equal volume of 0.5N HCl and 1ml of Arnow's reagent, 2ml of 1N NaOH and 4.5 ml of distilled water was added. The solution were mixed thoroughly (pink colour was appeared) and the absorbance (Visican-167) at 515nm was measured. The standard curve was

established using various concentrations of catechol. The ortho-dihydric phenols content was expressed as catechol equivalents (CLEs) in mg/g of dry material (Mahadevan and Sridhar, 1986).

Chromatographic separation

AARPE was subjected to thin layer chromatography using silica gel as stationary phase and hexane: acetone: ethyl acetate: (7:1:2) as mobile phase. The chromatograms when developed in iodine chamber yielded three spots. AARPE was subjected to repeated column chromatography to fractionate and isolate the compounds. 5.0g of AARPE was loaded in the column packed with silica gel (60-120 mesh size) in presence of hexane: EtOAc (99:1). The column was eluted first in hexane followed by gradient mode with varying percentage of EtOAc starting from 2% to 100%.

A total of 70 fractions were collected. Each fraction was monitored by TLC. Similar fractions were pooled together to yield eight fractions. Total 100 ml volume was collected in 2% EtOAc (fractions 1-14), 50 ml was collected in 5% EtOAc (fractions 15-20), 50 ml was collected in 10% EtOAc (fractions 21-25), 100 ml was collected in 15% EtOAc (fractions 26-35), 50 ml was collected in 20% EtOAc (fractions 36-41), 50 ml was collected in 25% EtOAc (fractions 42-46), 100 ml was collected in 25% EtOAc (fractions 47-52) and 150 ml was collected in 100% EtOAc (fractions 53-70). These fractions were further purified by re-column chromatography. The fractions obtained after recolumn were dried by removing the solvent in vacuum rotatory evaporator. The compounds finally crystallised in the form of crystals. Compound 1 and compound 2 was collected from column fraction 26-35 and 47-52 respectively.

Characterisation of compound 01: (Physiochemical data): Yield= 35mg, Physical character: White crystalline solid, Odour: Odourless, Solubility: Hexane, Ethyl acetate, Dichloromethane and methanol.

Characterisation of compound 02: (Physiochemical data): Yield=10mg, Physical character: White crystalline solid, Odour: Odourless, Solubility: Hexane and Ethyl acetate.

Results and discussion

Phytochemical screening

Fig. 1 shows the different spots on TLC with varying R_f values show different types of compounds. Further analysis of their TLC profiles show different types of phenolics, alkaloids, terpenoids and steroids present in various extracts of rhizomes of *A. allughas*.

Total flavonoid and orthodihydroxy phenol

The different flavonoid content found in the various extracts of *A. allughas* was determined spectrophotometrically based on the formation of flavonoid aluminium complexes which possess the maximum absorptivity at 510 nm. As depicted in the Table 1 the total flavonoid content of the extracts varied from 20.12±0.222 to 41.53±0.769 mg/g CNE (catechin equivalents) and AARME had the highest content of the flavonoids followed by AARDE>AARHE>AARPE in that decreasing order. The orthodihydric phenol content found in the various extracts ranged from 32.00±1.802 to 54.00±0.5 mg/g CLE (catechol equivalents) in which AARME had the highest orthodihydric phenol content followed by the other extracts.

Table 1. Total flavonoid and orthodihydric content of various extracts from rhizomes of *A. allughas*.

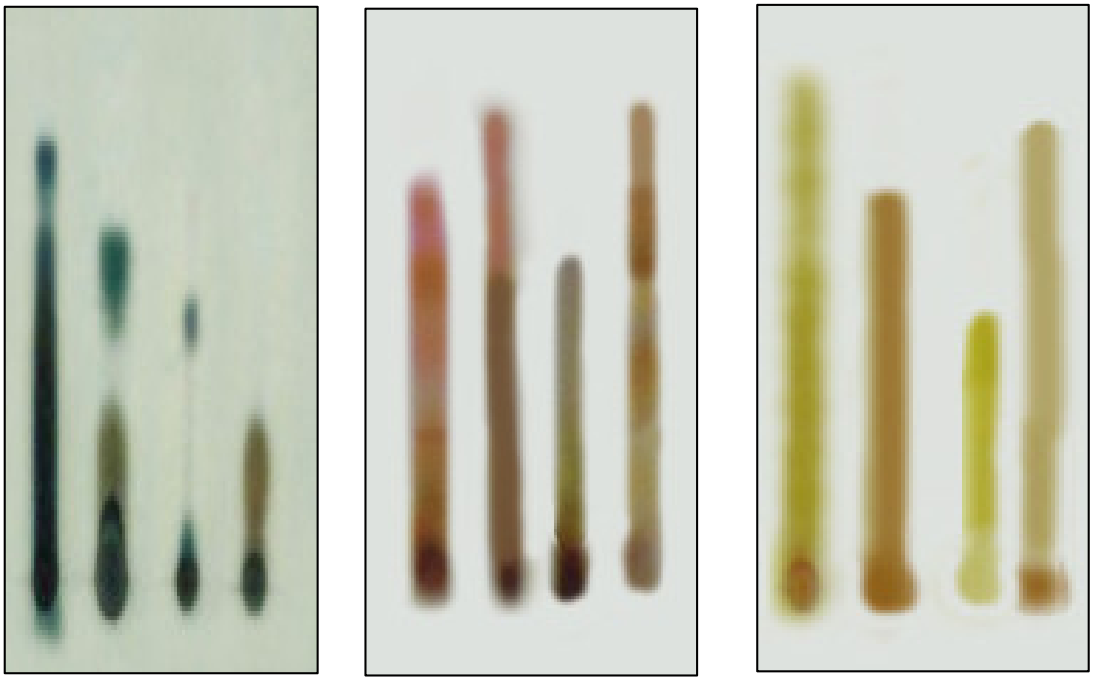
SN.	Sample name	Flavonoid (mg/g CNE)	Orthodihydric phenol (mg/g, CLE)
1	AARPE	20.12±0.222[a]	32.00±1.802[a]
2	AARHE	25.76±1.017[b]	41.50±0.866[b]
3	AARDE	35.76±0.384[c]	48.33±0.763[c]
4	AARME	41.53±0.769[d]	54.00±0.5[d]

- = *Not applicable*, Values are means of three replicates ± SD. Within a column, mean values followed by the same letter are not significantly different according to Tukey's test ($p<0.05$)

Column chromatography

The chromatographic separation and purification of AARPE yielded two compounds (compound 1 and compound 2) and the structure of these compounds was identified by IR, ^{1}HNMR and ^{13}CNMR as well as comparing the spectral data with the previously reported values.

Fig. 1: TLC profiles of various crude extracts from rhizomes of *A. allughas* showing (a) phenolic compounds in AARPE, AARHE, AARDE and AARME (b) steroids and terpenoids in AARPE, AARHE, AARDE and AARME (c) alkaloids in AARPE, AARHE, AARDE and AARME.



(a) **(b)** **(c)**

Table 2. ^{13}C and ^{1}HNMR data γ-Sitosterol and Stigmasterol (Measured in $CDCl_3$).

S. No.	γ-Sitosterol		Stigmasterol	
	^{13}C	^{1}H	^{13}C	^{1}H
1	37.25		37.25	
2	31.67		31.67	
3	71.83	2.285(m)	71.83	2.110(m)
4	42.30		42.30	
5	140.76		140.76	
6	121.76	5.380(s)	121.76	5.229(s)
7	31.92		31.92	
8	31.90		31.90	
9	50.11		50.11	
10	36.28		36.28	
11	21.09		21.09	
12	39.77		39.77	
13	46.03		46.03	
14	56.76		56.76	
15	24.32		24.32	
16	28.27		28.27	
17	56.30		56.03	
18	11.88	0.577(s)	11.88	0.629(s)
19	19.42	1.031(s)	20.10	1.212(s)
20	36.16		41.50	
21	18.79	0.947(d)	20.32	1.110(d)
22	33.93		135.43	
23	26.02		128.15	
24	45.81		51.63	
25	29.12		30.98	
26	19.85	0.850(d)	21.54	0.785(d)
27	19.03		19.03	
28	23.05		24.21	
29	12.00		12.00	

IR of 1st compound

The IR absorption spectrum showed absorption peak at 3440.77 cm^{-1} that corresponds to the presence of –OH group. The other fundamental peaks were peaks 2900 cm^{-1} for –CH stretching, 1644.59 cm^{-1} for –C=C stretching and 1413.69 cm^{-1} for $–CH_2$.

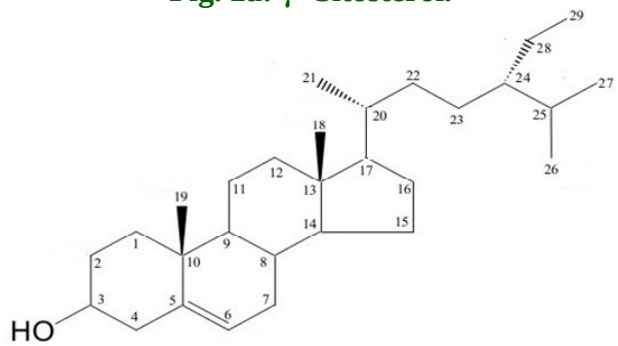
^{1}HNMR of 1st compound

^{1}HNMR has given signals at δ 2.285(m), 5.380(s), 0.577(s), 1.031 (s), 0.947 (d), 0.850 (d) ppm downfield to TMS. The 1HNMR spectrum ($CDCl_3$) of compound 1 indicated a one proton multiplet at 3.554 ppm. This position and multiplicity corresponds to the H-3 of steroid nucleus. A multiplet at 5.380 ppm corresponding to single hydrogen is indicative of the H-6 of the steroidal nucleus, and a doublet at 9.6ppm (Table 2).

^{13}CNMR of 1st compound

^{13}CNMR has given signal at 140.76 and 121.76 ppm for carbon atom 5 and 6 indicative of the double bond. β- hydroxyl group was shown by signal at 71.83 ppm at C3 carbon atom, 11.88 and 19.42ppm represent angular methyl carbon atoms for C18 and C19 respectively (Table 2). On the basis of these spectral data the compound was identified as γ- sitosterol (Fig. 2a). Further its identity was confirmed by the comparison of data with those recorded in literature (Tian et al., 2008; Pateh et al., 2009).

Fig. 2a: γ- Sitosterol.



IR of 2nd compound

The IR absorption spectrum showed absorption peak at 3426.65 cm^{-1} that corresponds to the presence of –OH group. The other fundamental peaks obtained were 1645.14 cm^{-1} for –C=C stretching, 1413.94 cm^{-1} for $–CH_2$ and 1106.46 cm^{-1} (cyclohexane) (Table 2)

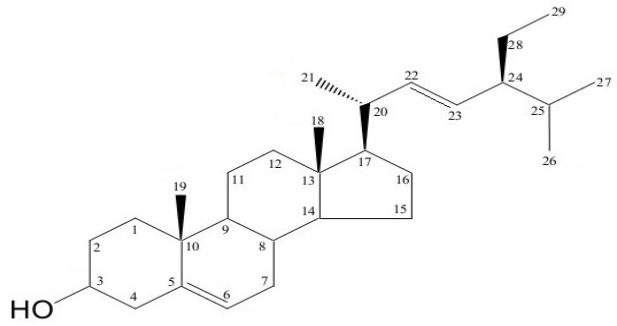
^{1}HNMR of 2nd compound

^{1}HNMR has given signals at δ 2.110(m), 5.229(s), 0.629(s), 1.212 (s), 1.110 (d), 0.785 (d) ppm downfield to TMS. The ^{1}HNMR spectrum ($CDCl_3$) of compound 2 indicated a one proton multiplet at position 3.365 ppm whose multiplicity corresponds to the H-3 of steroid nucleus. A multiplet at 5.229 ppm corresponding to single hydrogen is indicative of the H-6 of the steroidal nucleus, and a doublet at 9.6 ppm (Table 2).

^{13}C-NMR of 2nd compound

^{13}CNMR has given signal at 140.76 and 121.76 ppm for carbon atom 5 and 6 indicative of the double bond. Signal at 71.83 ppm at C3 carbon atom indicative of β-hydroxyl group. However signals at 11.88 and 20.10 ppm represent angular methyl carbon atoms for C18 and C19 respectively (Table 2). ^{13}C spectra showed the presence of 29 carbon atoms. The above features are in close agreement to those observed for stigmasterol (Fig. 2b) in literature (Tian et al., 2008; Kamboj and Saluja, 2011).

Fig. 2b: Stigmasterol.



Acknowledgement

Authors are thankful to the DST- INSPIRE and University grant commission (UGC), New Delhi, Govt. of India for financial assistance. J.N.U., New Delhi is highly acknowledged for the spectral analysis of data. The research work was carried out at G.B.P.U.A & T, Pantnagar.

References

Aguin, O., Mansilla, J.P., Maria, J.S., 2006. *In vitro* selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapewine in the field. Pest Manag. Sci. 62, 223-228.

Bungihan, M.E., Matias, C.A., 2013. Determination of the antioxidant, phytochemical and antibacterial profiles of flowers from selected ornamental plants in Nueva Vizcaya, Philippines. J. Agric. Sci. Tech. 3, 833-841.

Choi, Y., Lee, S.M., Chun, J., Lee, H.B., Lee, J., 2006. Influence of heat treatment on antioxidant activities and polyphenolic compounds of Shiitake (*Lentinus edodes*) mushroom. Food Chem. 99, 381-387.

Chunekar, K.C., 1982. Bhavaprakashanighantu of Sri Bhavamishra. Commentary, Varanasi (in Hindi).

Daayf, F., Schmitt, A., Belanger, R.R., 1995. The effects of plant extracts of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. Plant Dis. 79, 577–580.

Ghani, A. 2003. Medicinal plants of Bangladesh with chemical constituents and uses. 2nd ed. Asian Society of Bangladesh, Dhaka, Ramna. 42p.

Jaracz, S., Malik. S., Nakanishi, K., 2004. Isolation of ginkgolides A, B, C, J and bilobalide from *G. biloba* extracts. Phytochem. 65, 2897-2902.

Kamboj, A., Saluja, A.K., 2011. Isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). Int. J. Pharm. Pharm. Sci. 3(1), 94-96.

Karthikeyan, S., 2000. A statistical analysis of flowering plants of India, In: Flora of India introductory volume (Part II) (Eds.: Singh, N.P., Singh, D.K., Hajra, P.K., Sharma, B.D.). Botanical Survey of India Calcutta. pp. 201-217.

Kirtikar, K.R., Basu, B.D., 1987. Indian Medicinal Plants. Internat Book Distributors, Dehradun. pp. 2444-2449.

Mahadevan, A., Sridhar, R., 1986. In: Methods in Physiological Plant Pathology. 3rd Edn. Sivakami Publication, Chennai. pp. 190-192.

Pateh, U.U., Haruna, A.K., Garba, M.,Iliya, I., Sule, I.M., Abubakar, M.S., Ambi, A.A., 2009. Isolation of stigmasterol, β-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton lancifolius* Pyer and Kotchy (Araceae). Nig. J. Pharm. Sci. 1, 19-25.

Prakash, V., Mehrotra, B.N., 1995. Zingiberaceae of North-East India: Diversity and taxonomic status. Proc. 2nd Symp. Family Zingiberaceae. pp. 262–273.

Sethi, S., Prakash, O., Punetha, H., Pant, A.K., 2013. Antifungal activity of essential oils of some *Ocimum* species collected from different locations of Uttarakhand. Indian J. Nat. Prod. Resour. 4(4), 392-397.

Tian, M.Q., Bao, G.M., Ji, N.Y., Li, X.M., Wang BG., 2008. Triterpenoids and steroids from *Excoecaria agallocha.* Zhongguo Zhong Yao Za Zhi. 33(4), 405-408.

Warrier, P.K., Nambiar V.P.K., Ramankutty, C., 1993-1995. Indian Medicinal Plants. Orient Longman Ltd., Madras.