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Original Research Article

Isolation of squarrosal and squarrosol compounds from methanol root extract of *Ruellia squarrosa* (Acanthaceae)

Khurram Afzal[1,2]*, Muhammad Uzair[1], Bashir Ahmad Chaudhary[1], Saeed Akhtar[2], Ashfaq Ahmad[3*], Samina Afzal[1]

[1]Faculty of Pharmacy, [2]Institute of Food Science and Nutrition, Baha u din Zakariya University, Multan, Pakistan, [3]Department of Pharmacology and Toxicology, School of Medicine, Virginia Commonwealth University, Richmond 23298, USA

For correspondence:* ***Email:* *khurram.afzal@bzu.edu.pk*

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Abstract

Purpose: *To identify, characterize and structurally elucidate bioactive compounds from root of Ruellia squarrosa.*
Methods: *One kilogram of crude Ruellia squarrosa root was shade dried for 14 days, ground to a fine powder and subjected to a methanol extraction. The resultant extract underwent column chromatography for further purification. The isolated compounds were subjected to ultraviolet spectroscopy (UV), infra-red (IR), proton nuclear magnetic resonance (^{1}H-NMR), ^{13}C–NMR and high resolution electron ionization mass spectrometry (HR-EI-MS) for the identification, characterization and structural elucidation of bioactive compounds. The most active compounds were tested for anticancer activities against human prostate cancer cell.*
Results: *Two active compounds, squarrosol and squarrosal, were obtained with half-maximal inhibitory concentration (IC_{50}) of 15.6 and 26.6 µg/mL, respectively, against human prostate cancer cell lines. Squarrasol showed a significantly ($p < 0.05$) greater inhibition of cell proliferation than the same dose of squarrasal.*
Conclusion: *These findings suggest that extracts of Ruellia squarrosa containing the bioactive compounds, squarrosol and squarrosal, can potentially be developed for the treatment of human prostate cancer.*

Keywords: *Ruellia squarrosa, Prostate cancer, Squarrosol, Squarrosal, Anit-proliferative*

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INTRODUCTION

Medicinal plants have made a great impact on human health and extracts have been used for treatment of various diseases and have been found to have antimicrobial activity and antiviral activity [1-3]. Researchers have turned their attention towards herbal products as potential sources in their search for more effective drugs in the treatment of cancer, cardiac and infectious diseases [4,5]. Since ancient time, plants and plant extracts have been used traditionally as medicinal remedies and it is estimated that 80-85 % of the world population relies on herbal plants and extracts for medicinal purposes [6].

The genus *Ruellia*, generally known as *Ruellias* or Wild Petunias, belongs to the family Acanthaceae. It includes about 250 genera and 2500 species. Most of these are shrubs or twining vines; some are epiphytes, with most species occurring in tropical regions and only a few being distributed in temperate regions. They are found in Indonesia, Malaysia, Africa, Brazil, Central America and Pakistan. They are represented in Pakistan by 5 species, of which 3 are native [7]. Extracts of different species of genus *Ruellia* have been reported to possess anti-inflammatory, anti-pyretic [8], antiulcer [9], antiproliferative [10] and antidiabetic activities [11]. The plant *Ruellia squarrosa* has been used traditionally for the treatment of stomach cancer. Dried root extracts of the plant has been used used to induce abortions and for inflammation in the eyes [12].

The present investigation was conducted in order to extend previous observation [13], in order to isolate the active compounds from plant *Ruellia squarrosa*and and evaluate their anticancer potential against prostate cancer cell lines by measuring their IC_{50} values and % inhibition of proliferation in human prostate cancer cell lines.

EXPERIMENTAL

Plant collection

The plant *Ruellia squarrosa* was collected from the University Garden of Bahauddin Zakariya University (B.Z.U), Multan (District in Punjab, Pakistan) in August, 2013 and identified by Prof. Dr. Altaf Ahmad Dasti, Plant Taxonomist, Institute of Pure and Applied Biology, B.Z.U Multan, Pakistan. A reference specimen (fl.c.1-2) was deposited in the Institute of Pure and Applied Biology B.Z.U Multan.

Extraction

Roots of *Ruellia squarrosa*, 500 g, were shade dried for two weeks and then powdered by using homogenizer. The fine powder was subjected to extraction by mixing with one liter volume of methanol at room temperature for 24 hours. The methanol extract was filtered and dried by rotavapor - R200 at 35^{o}C until semisolid appearance of extract was observed in collection flask. A total amount of 35 g of extract of *Ruellia squarrosa* was collected in collection flask.

Isolation procedure

The methanolic extract, about 8g, was fractionated using silica gel 60 column chromatography and step wise elusion with a mixture of chloroform: methanol: water (70:30:4 v/v/v) in increasing order of polarity. Six fractions were obtained using this approach. Fraction 3 containing, 370 mg, was further fractionated using silica gel 60 column chromatography but with stepwise elution using a ethyl acetate: methanol: water (98: 14: 7 v/v/v) respectively. As a result, 9 sub-fractions were obtained from which two pure compounds were isolated namely compound squarrosal, approximately 6 mg, and squarrosol, of approximately 5 mg.

General

For TLC purposes, aluminum sheets pre-coated with silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E-Merck) were used to check the percentage purity of the compounds. The visualization of components took place under ultraviolet light (254 and 366 nm) followed by Godine reagent and 10% sulphuric acid as a spraying reagent. Column chromatography for isolation purposes used silica gel, 70-230 mesh, and 230-400 mesh along with sephadex LH-20. An IR spectrum was recorded using a Bruker vector-200 spectrophotometer (v in cm^{-1}). The ^{1}H-NMR spectrum was measured using a Bruker Avon-300 MHz instrument with TMS as an internal standard. The chemical shift values were reported in ppm (δ) units and the coupling constants (*J*) are recorded in Hz. The ^{13}C -NMR spectrum was also recorded using the Bruker Avon-300 MHz instrument. EI-MS spectrum was measured using a Jeol JMS-600H spectrometer and HREI-MS with a MET-95-XP.

Solvents and materials

Methanol, dichloromethane, chloroform, n-hexane, ethyl acetate, ethanol, propanol, *n*-butanol Vanillin, silica gel (70-230 mesh), TLC aluminum sheets (20 x 20) cm and Silica gel 60 F_{254} were imported from Merck (Darmstadt Germany). Sephadex LH-20 25 - 100 um and Fluka Chemie GmbH (9041-37-6).

Chromatographic studies

The separation and purification of the plant constituents relied mainly on different chromatographic techniques, the choice of which depended upon the property and volatility of compound to being identified or separated.

Biological application of these compounds on prostate cancer cell lines

An MTT assay was performed as reported previously [13]. Briefly, human prostate cancer

cells (ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5 % foetal bovine serum were kept at 5 % incubator at 37 ^{0}C.

Growing cells were cultivated, harvested and diluted with a specific medium. (5 – 10) x 10^4cells/well were treated for 48 h with six different concentrations of squarrosol and squarrosal in µg/mL (2.5 - 25 ug/ml). 0.5 % dimethyl sulfoxide (DMSO) and 5 fluorouracil (5-FU) were used as negative and positive control substances, respectively. Absorbance was read using a microplate reader (TECAN Infinite Pro®M200, Switzerland) at 570 nm using 620 nm as the reference wavelength.

Statistical analysis

The results presented are mean ± SD. One-way ANOVA was performed to test differences between groups which were considered significant at $p < 0.05$. GraphPad Prism (Graph PAD, San Diego, USA) was used for statistical analysis.

RESULTS

Physical and spectroscopic characteristics

Squarrosal

UV (Me OD) λ_{max}logε nm: 212 (1.2); IRu_{max} (KBR) cm^{-1}: 2926, 2856, 1739, 1593, 1452, 1184, 1132, 860; ^{1}H-NMR (DMSO, 500MHz): δ7.93 (1H, s , H-1),7.92 (1H, d, *J* = 7.0 , H-2,H-3),7.24 (1H, d, *J* = 6.8 , H-2,H-4),1.58(1H, t, *J* = 2.8, H-2, H-7),6.47(1H, s, H-9), 2.69(2H, q, *J* = 6.4, H-2,H-12),0.74(3H, t, *J* = 2.5, H-13),1.16(2H, dt, *J* = 2.9,3.7, H-14),1.15(2H, dt, *J*=2.0,H-15), 1.69(1H, m, H-16),6.16(1H, dd, *J* = 6.0, 5.0, H-17), 6.18 (1H, dd, *J*=6.8,5.1, H-18), 6.45(1H, dd, *J* = 5.9, 7.0, H-19),4.42(1H, dd, *J*= 6.1, 6.8, H-20),5.55(1H,d,*J*=7.8,H-21)3.84 (1H,dd,*J*=7.2,5.0,H-22) 3.61 (1H,dd, *J*=5.7,3.9, H-23) 3.60(1H,dd, *J*=7.3,4.7, H-24), 6.12(1H,d, *J*=6.5, H-25); ^{13}C-NMR ($CDCl_3$, 125MHz): δ 128.0 (C-1), 130.0 (C-2), 125.0 (C-3), 124.0 (C-4), 131.0 (C-5), 167.0 (C-6), 50.0 (C-7), 115.0 (C-8), 142.0 (C-9), 137.0 (C-10), 141.6 (C-11), 67.9 (C-12), 63.0 (C-13), 23.7 (C-14), 39.7(C-15), 29.3 (C-16), 154.6(C-17)131.3 (C-18), 131.0(C-19), 154.0 (C-20), 92.5 (C-21), 77.2 (C-22)77.0 (C-23), 76.7(C-24), 97.6(C-25): EI-MS *m/z* (rel. int): 413(400), 219(240), 166(243); HR-EI-MS *m/z*: 456 [M+H]$^+$ (calculated for $C_{25}H_{28}O_8$: 456).

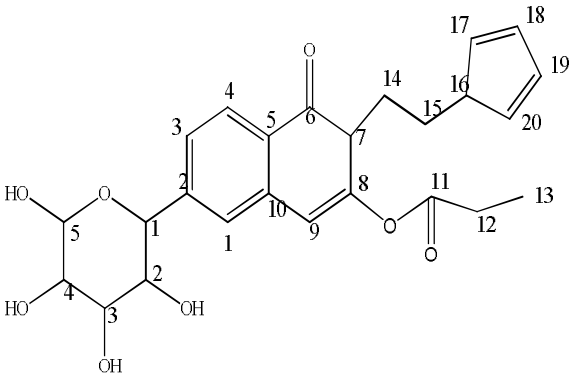

Figure 1: Structure of squarrosal

Squarrosal was isolated from the methanol fraction of *Ruellia squarrosa* as an amorphous powder. The infrared spectrum of the compound had absorption bands at 1739 cm^{-1}due to lactone group. A band was observed at 1593 cm^{-1} due to aromaticity. The stretching vibrations at 2856 cm^{-1} showed the presence of sp^3C−H and at 2925 cm^{-1} showed the presence of Sp^2 C−H.

The ultraviolet visible spectrum of the compound demonstrated an absorption band at 212 nm. The molecular formula was inferred as $C_{25}H_{28}O_8$ through the JEOLJMS-600H presenting a molecular ion peak [M+H] at m/z 456 (calculated for $C_{25}H_{28}O_8$:456) (Table 2). The mass fragmentation of the compound is given in Figure 1:

The ^{1}H-NMR spectrum of squarrosal showed a signal for an alcoholic group at δ3.61 (1H, dd, *J* = 3.9, 5.7 Hz), 3.84 (1H, dd, *J* = 5.0, 7.2 Hz) and 3.60 (1H, dd, *J* = 4.7, 7.3 Hz). The aliphatic ring protons appeared at δ 2.94 (2H, d, *J* = 2.6 H), 1.16 (2H, dt, *J* = 2.1, 3.7 Hz); the methyl group appeared at δ 0.74 (3H, t); and the lactone hydrogen at δ 5.55 (1H, d, *J* = 7.8, Hz).

The ^{13}C-NMR spectrum (BB and DEPT) of squarrosal exhibited 25 carbon signals for one methyl, fifteen methines, four methylenes and five quaternary carbon atoms. The downfield signal at δ 167.6, 141.6 showed the presence of carbonyl carbon group. The lactone ring appeared at δ92.5 and 97.6; the aliphatic carbon appeared at δ1.176 to 2.94; and the alcoholic peak was observed at δ 77.2, 70.0 and 76.7.

On the basis of these data, the structure was established as 2-(2-(cyclopenta-2,4-dien-1-yl)ethyl)-3-(2-oxopropyl)-6-(3,4,5,6 tetrahydroxytetrahydro-2H-pyran-2-yl)-3,4-dihydronaphthalen-1(2H)-one and it was found to be a novel natural product. It was named on the basis of the species as squarrosal.

Table 1: ^{13}C-NMR and chemical shift assignments of squarrosal

Carbon no.	DEPT	^{13}C-NMR	^{1}H-NMR	*J.* Value
C – 1	CH	128.0	7.93 (s)	-
C – 2	C	130.0	-	-
C – 3	CH	125.0	7.92 (d)	7.0
C – 4	CH	124.0	7.24(d)	6.8
C – 5	C	131.0	-	-
C – 6	C	167.0	-	-
C – 7	CH	50.0	1.58(t)	2.8
C – 8	C	115.0	-	-
C – 9	CH	142.0	6.47(s)	-
C – 10	C	137.0	-	-
C – 11	C	141.6	-	-
C – 12	CH_2	67.9	2.69(q)	6.4
C – 13	CH_3	63.0	0.74(t)	2.5
C – 14	CH_2	23.7	1.16(dt)	2.9, 3.7
C – 15	CH_2	39.7	1.15(dt)	2.0, 4.5
C – 16	CH	29.3	1.69(m)	-
C – 17	CH	154.6	6.16(dd)	6.0,5.0
C- 18	CH	131.3	6.18(dd)	6.8, 5.1
C- 19	CH	131.0	6.45(dd)	5.9,7.0
C- 20	CH	154.0	4.42(dd)	6,1,6.8
C- 21	CH	92.5	5.55(d)	7.8
C- 22	CH	77.2	3.84(dd)	7.2,5.0
C- 23	CH	77.0	3.61(dd)	5.7,3.9
C- 24	CH	76.7	3.60(dd)	7.3,4.7
C- 25	CH	97.6	6.12(d)	6.5

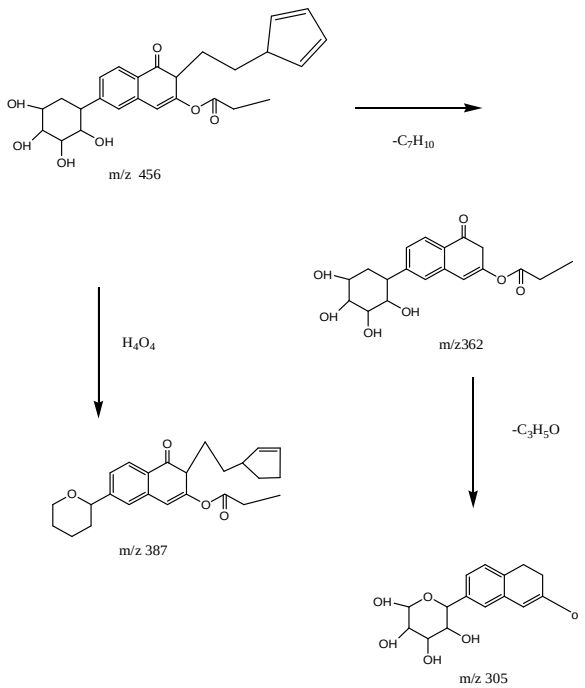

Figure 2: Mass fragmentation of compound squarrosal

Squarrosol

Non crystalline solid (05 mg); UV (Me OD) λ_{max}logεnm: 208 (0.65), 284 (0.14); IRu$_{max}$ (KBR) cm^{-1}: 3463, 3060; ^{1}H-NMR (DMSO, 500MHz): δ7.65 (1H, s, H-1), 7.67 (1H, s, H-3), 4.40 (1H, d, *J*= 7.0, H-8), 4.45 (1H, d, *J*= 6.8 H-9), 7.46 (1H, d, *J*= 7.2, H-12), 7.48 (1H, d, *J* = 6.8, H-13), 7.70 (1H, s, H-15), 0.86 (3H, s, H-16), 7.65 (1H, s, H-19), 7.67 (1H, s, H-21), 6.9 (1H, s, H-24), 6.43 (1H, m, *J*=6.8, H-26), 4.39 (2H, d, *J* = 7.8, H-27), 4.37 (3H, s, H-28); ^{13}C-NMR ($CDCl_3$, 125MHz): δ131.8 (C-1), 131.7 (C-2), 129.2 (C-3), 132.4 (C-4), 109.5 (C-5), 140.0 (C-6), 167.5 (C-7), 128.7 (C-8), 128.6 (C-9), 116.8 (C-10), 134.5 (C-11), 116.3 (C-12), 121.8 (C-13), 128.7 (C-14), 115.8 (C-15), 22.2 (C-16), 164.4 (C-17), 102.3 (C-18), 131.7 (C-19), 117.6 (C-20), 118.6 (C-21), 114.2 (C-22), 131.8 (C-23), 72.5 (C-24), 132.8 (C-25), 116.8(C-26), 135.6(C-27), 60.8(C-28); EI-MS *m/z:* 484(10), 443(20), 399 (34), 354(30), 132 (42), 89(62), 65(76) HR-EI-MS m/z: 484 [M+H]$^{+}$ (calculated for $C_{28}H_{24}N_2O_6$: 484)

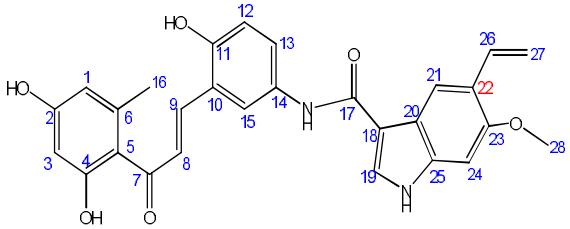

Figure 3: Structure of squarrosol

Squarrosol was isolated as a noncrystalline solid from the methanolic extract of *Ruellia squarrosa*. In the Infrared spectrum of the Squarrosol compound, the absorption bands at 1726 cm^{-1} was consistent with the presence of a carbonyl functional group. The stretching vibrations at 34.63 cm^{-1} suggested the presence of Sp^2 C−H. The ultraviolet visible spectrum displayed absorption peaks at 208 and 284 nm. The molecular formula was inferred as $C_{28}H_{24}N_2O_6$ using the JEOLJMS-600H which displayed a molecular ion peak [M+H] at m/z 484 (calculated for $C_{28}H_{24}N_2O_6$:484) (Table 3).

The ^{1}H-NMR spectrum of the Squarrosol compound gave a signal for aromatic ring protons δ 7.67 (1H, s), 7.41 (1H, d, *J* = 6.5 Hz), 7.69 (1H, d, *J* = 6.8Hz) 7.46 (1H, d, *J* = 7.2 Hz), 7.48 (1H, d, *J* = 6.8 Hz), 7.65 (1H, s) and 7.67 (1H, s). The alkene proton showed doublet and multiplets at δ 4.40 (1H, d, *J* = 7.0 Hz) 4.45 (1H, d, *J* = 6.8 Hz) and 4.39 (1H, d, *J* = 7.8 Hz).

The ^{13}C- NMR spectrum (BB and DEPT) of the Squarrosol compound was consistent with a total of 28 carbon signals for two methyl, eleven methines, one methylene and 14 quaternary carbon atoms. The downfield signal at δ 167.5, 163.1, 161.7 revealed the presence of a C = O group. The downfield signal appeared at δ 140.0,

135.6, 131.8 indicating the presence of an aromatic functional group.

The compound structure was established as (E)-N-(3-(3-(2, 4-dihydroxy-6-methylphenyl)-3-oxoprop-1-enyl)-4-hydroxyphenyl)-6-methoxy-5-vinyl-1H-indole-3-carboxamide. On the basis of the above data, the compound was identified as a novel natural product and named on the bases of the species as squarrosol.

Table 2: ^{13}C-NMR chemical shift assignments of squarrosol

Carbon no.	DEPT	^{13}C-NMR	^{1}H-NMR	*J*.value
C – 1	CH	131.8	7.65(s)	-
C – 2	C	131.7	-	-
C – 3	CH	129.2	7.67 (s)	-
C – 4	C	132.4	-	-
C – 5	C	109.5	-	-
C – 6	C	140.0	-	-
C – 7	C	167.5		-
C – 8	CH	128.7	4.40(d)	7.0
C – 9	CH	128.6	4.45 (d)	6.8
C – 10	C	116.8	-	-
C – 11	C	134.5	-	-
C – 12	CH	116.3	7.46 (d)	7.2
C – 13	CH	121.8	7.48(d)	6.8
C – 14	C	128.7	-	-
C – 15	CH	115.8	7.70(s)	-
C – 16	CH_3	22.2	0.86 (s)	-
C – 17	C	164.4	-	-
C – 18	C	102.3	-	-
C – 19	CH	131.7	7.65(s)	-
C – 20	C	117.6	-	-
C – 21	CH	118.6	7.67 (s)	-
C – 22	C	114.2	-	-
C – 23	C	131.8	-	-
C – 24	CH	72.5	6.91 (s)	-
C – 25	C	132.8	-	-
C – 26	CH	116.8	6.43 (m)	-
C – 27	CH_2	135.6	4.39(d)	7.8
C – 28	CH_3	60.8	4.37(s)	-

Effect of squarrosal and squarrosol of cell proliferation

The results were presented as percent viability compared to the negative control (mean ± SD, n=3). IC50 values were calculated using the anti-proliferation activity of different concentrations of squarrosol and squarrosal as 15.6 and 26.6 µg/mL respectively. Dose of dependent inhibition on prostate cell proliferation is shown in Figure 4.

DISCUSSION

The present study was based on the isolation of phytochemical constituents from the herbal plant *Ruellia squarrosa* and evaluation of active compounds for their anti-proliferative activity on

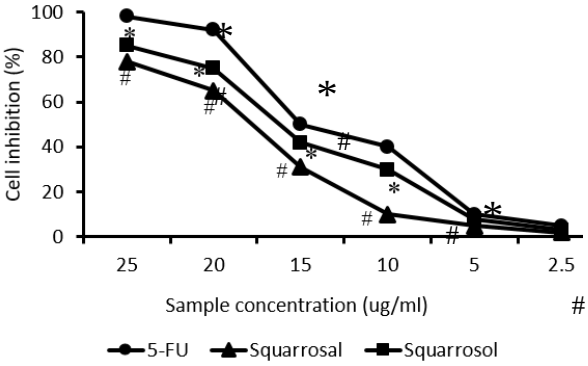

Figure 4: Dose-dependent activity of squarrosal and squarrosol on prostate cell proliferation. **P* < 0.05 vs. ose of squarrosal; #*p* <0.05 vs. dose of 5-FU

human prostate cancer cell lines. The present investigation isolated two novel compounds from methanol extract of *Ruellia squarrosa* which belong to the flavonoid class and were named squarrosal and squarrosol. Their anticancer potential against human prostate cancer cell lines was explored as this feature has been established with other flavonoids [14]. This study reported herein represents a continuation of our previous finding [13] in which it was found that extracts of different parts of the *Ruellia squarrosa* plant contain compounds which possess anticancer potential against human prostate cancer lines. However, the previous study was unable to isolate the active compounds. The current investigation set out to isolate the active constituents of *Ruellia squarrosa*. It was found that squarrosol and squarrosal both exhibited dose dependent inhibition of proliferation of the prostate cancer cells. Squarrosol exhibited a significantly greater ($p < 0.05$) % inhibition of proliferation against prostate cancer cell lines when compared to that of squarrosal. However, this activity was less than the degree of inhibition of prostate cancer cell proliferation using the standard drug, 5-FU. These flavonoids are amongst the few medicinal plants which have been reported to have anticancer potential and this activity is in line with the flavones which have been reported to have broad activity against tumors, leukemia, lymphomas and solid tumors [15]. This study is also in line with recent study [13] which demonstrated that flavonoids may be potential therapeutic compounds for the treatment of cancer.

CONCLUSION

These findings show that squarrosal and squarrosol are the main active compounds contained in the extracts of *Ruellia squarrosa* and exert antiproliferative effect on human

prostate cancer cells. Thus, these compounds can potentially be developed for human prostate cancer therapy.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Khurram Afzal and Muhammad Uzair contributed equally to the work.

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