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ANTIBACTERIAL ACTIVITIES OF THE METHANOL EXTRACT AND FRACTIONS OF THE LEAF OF *ERIOSEMA PSORALEOIDES* (LAM.) G. DON (LEGUMINOSAE)

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Keywords:

Eriosema psoraleoides, Antibacterial, MIC, Staphylococcus aureus

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ABSTRACT: Eriosema psoraleoides leaves have been reported to be used in traditional African medicine to treat cuts and wounds, hence the present investigation of its antibacterial activity using the agar pour plate method. The leaves contain tannins and flavonoids. A methanol extract was obtained from the leaves by maceration, and then the n-hexane and ethyl acetate fractions were obtained from this. Statistical analysis of data was by ANOVA, with p<0.05 considered significant. The methanol extract, nhexane fraction and ethyl acetate fraction were found active against Staphylococcus aureus ATCC 25923, and Staphylococcus aureus Clinical isolate; in addition, the methanol extract was active against Pseudomonas aeruginosa. However, Staphylococcus aureus Clinical isolate was found resistant to Gentamicin. The Minimum Inhibitory Concentrations (MICs) of the extract and fractions against Staphylococcus aureus ATCC and against Staphylococcus aureus Clinical isolate, showed that ethyl acetate fraction was more active than the other two fractions. Although Staphylococcus aureus was found to be resistant to Gentamicin, it was susceptible to the antibacterial activity of the methanol extract, n-hexane fraction, and ethyl acetate fraction of the plant. This suggests that these extract/fractions may be useful in treating infections caused by Staphylococcus aureus.

INTRODUCTION: The fact that pathogenic micro-organisms do develop resistance against antimicrobial drugs, leading to treatment failures and complications, has made it necessary for the search for new drugs. The World Health Organisation, WHO, reports that "resistance to the first-line drugs used to treat infections caused by *Staphylococcus aureus* – a common cause of severe infections acquired both in health-care facilities and in the community – is also widespread" ¹.



Medicinal plants have proven to be a very viable source of new compounds that can be formulated into drugs. So, in the search for newer and more effective antimicrobial agents, medicinal plants represent a natural choice ²⁻⁶. Drugs obtained from natural sources which are being used to fight cancer, malaria cardiovascular and metabolic diseases include statins and artemisinin⁷⁻⁹.

The plant under the present study, *Eriosema* psoraleoides (Lam) G.Don (Leguminosae), has been reported to be used as chewing stick for the cure of dental problems in ethnomedicine ¹⁰. In another report, *E. psoraleoides* is said to be used in a polyherbal preparation to treat malaria in Tanzania; and that the stem bark of *Sapium* ellipticum, and root of *Vernonia amygdalina* are included in this preparation ¹¹.

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An earlier published report had indicated that E. psoraleoides is used both as antimalarial and aphrodisiac 12 .

Further, the whole plant is said to be powdered and taken to clear tuberculosis, in Tanzania. The roots, leaves and fruits are used to treat injuries to the foot ¹³. So, the purpose of this study is to find out if this plant has antibacterial activity, as this activity has not been found reported for it in the literature.

Description: Erect undershrub, grey-silky, more or less branched, 4-5ft high or more in tall grass; flowers are golden yellow and odorous. It is common throughout tropical Africa extending to South Africa ¹⁴.

The plant has several synonyms which include *Eriosema macrophyllum* Klotzsch; *Eriosema procumbens* Benth. Ex Baker; *Rhynchosia cajanoides* Guill. & Perr., and *Crotalaria psoraloides* Lam. Its English common names are Canary Pea and Yellow Seed.

Reported activity: The genus Eriosema has about 150 species ¹⁵. Among these, some of the investigated species include E. tuberosum, E. kraussianum, E. glomerata and E. englerianum. The essential oil (from the fresh leaves) of E. englerianum showed anti-oxidant activity, and good antibacterial and antifungal activities 16. Antimicrobial prenylated dihydro-chalcones have been isolated from E. Glomerata 17. Two pyranocompounds extracted from isoflavone kraussianum have been reported to exhibit vasodilatory and hypoglycaemic activities ¹⁸; also, from the same plant, have been isolated pyroisoflavones showing erectile-dysfunction corrective activity ¹⁹. Polyphenols ²⁰ and four chromones have been reportedly extracted from E. Tuberosum ²¹.

MATERIALS AND METHODS: The solvents used for this study, methanol, ethylacetate, n-hexane, and chloroform were all Sigma-Aldrich brands. The Nutrient Agar and Nutrient broth were Mueller-Hinton brands. The plant material was sourced from Nsukka in the South Eastern part of Nigeria, during the peak of the rainy season in June. Authentication was done by Mr. Alfred Ozioko of the Biodiversity and Conservation Programme (BDCP), Nsukka. The plant herbarium number is INTERCEDD 968.

Processing of plant material: Fresh leaves of the *Eriosema psoraleoides* plant were harvested and the leaves separated from the stem. The leaves were air dried under shade, for two weeks. The dried leaves were pulverized to obtain a coarse powder form which was used for the extraction by maceration.

Extraction: a 300gm of the powdered plant material was placed in a stoppered amber-coloured glass container and methanol was poured in to a level above the powder and was allowed to stand at room temperature for 72hrs, with frequent agitation. The mixture was then filtered, and to the marc was added fresh solvent and kept for another 48hrs with frequent agitation. The filtrate was obtained with the aid of a Buchner funnel and Whatman No.1 filter paper. The combined filtrate was then concentrated under reduced pressure in a rotary evaporator at 40 °C. This methanol extract was subsequently dried in desiccators, packaged and preserved in a refrigerator until ready to be used.

Fractionation: In order to obtain the n-hexane and ethyl acetate fractions, the methanol extract was first washed exhaustively with three portions of 20ml each of n-hexane in succession, and the washings were combined to obtain the n-hexane fraction. Then, the procedure was repeated with ethyl acetate to get the ethyl acetate fraction, and then the ethyl acetate insoluble.

Phytochemical screening: The methanol extract, n-hexane and ethyl acetate fractions were screened for the presence of alkaloids, saponins, tannins, phlobatannins, flavonoids, anthraquinones, and deoxysugars using methods described by Evans ²². For the Thin Layer Chromatographic analyses of the extract and fractions, TLC plates precoated with silica gel 60F254 were used, and the chosen solvent system was chloroform: n-hexane (4:2). Detection of spots was by spraying plates with ferric chloride, iodine vapour and DPPH, and viewing under UV lamp.

Microbiological test: The micro-organisms used for this study were *Escherichia coli* ATCC 35219; *E. coli* clinical isolate; *Staphylococcus aureas* ATCC 25923; *S. aureas* clinical isolate; *Proteus vulgaris* clinical isolate; *Klebsiella aerogenes* and

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Pseudomonas aeruginosa clinical isolate. The standard organisms were obtained from the Nigerian Institute of Medical Research (NIMR), and the clinical isolates from the University of Port Harcourt Teaching Hospital. Stock cultures of these organisms were made on agar slant of nutrient agar, and stored at 50°C, the innocula for the experiments were obtained by sub-culturing this stock culture.

Nutrient broth and nutrient agar media were prepared with powdered agar (Muller Heintein), using standard procedures, and then sterilized by autoclaving at 121°C for 15mins. Bacteria cultures were prepared by transferring a loopful of each of the bacterial colonies from the agar slant into separate tubes containing 10ml of sterile nutrient broth. The tubes were then incubated at 37°C temperature for 24hrs.

Sensitivity testing of the extract and fractions on pathogenic bacteria: The methanol extract, ethyl acetate and n-hexane fractions were dissolved in dimethylsulfuroxide (DMSO) to obtain concentration of 10mg/ml of the extract and fractions. A standard solution of Gentamicin. 4ug/ml, was also prepared. Ten of the nutrient agars in bijou bottles prepared previously were melted cooled to about 40°C and each was seeded with 1ml of a 1:10 dilution of the overnight culture of each test organism; the molten agar was then poured into different sterile petri-dishes and allowed to set. Using a sterile 6mm cork borer, wells were made in the seeded plates aseptically and the plugs of the agar obtained removed with a flamed loop. The resulting wells were then labeled and filled with the fractions; the tests were performed in triplicates. The solution Gentamicin and dissolving solvent (DMSO) were also filled into separate wells. The plates were left for an hour to allow diffusion of the fraction into the agar after which they were incubated at 37°C for 24hrs. The zones of inhibition produced by the extract and fractions were then measured and compared with those of the standard and DMSO.

Determination of the Minimum Inhibitory Concentration (MIC) of the extract and fractions: The extract and fractions were dissolved in DMSO to obtain 10mg/ml, 8mg/ml, 6mg/ml, 4mg/ml and 2mg/ml of each. These dilutions of the extract and

fractions were put into wells of agar medium which had been inoculated with Staphylococcus aureus ATCC 25923, and S. aureus clinical isolate: whereas, only the methanol extract dilutions were put into wells of agar seeded with Pseudomonas aeruginosa, as a result of the sensitivity test earlier carried out. The plates were incubated at 37°C for 24hrs. The zones of inhibition after 24hrs. were measured and a plot of Inhibition Zone Diameter (IZD²) versus squared logarithm of concentration was done to determine the MIC of each extract. The value of the MIC was obtained by computing the antilogarithm of the intercept of the linear graph (line of best fit) on the x-axis (in $mg/ml)^{23,24}$.

Statistical Analysis: Experiments were conducted in triplicates, and the values presented as their mean \pm standard deviation. Analysis was done with ANOVA with the significance level at p<0.05.

RESULTS AND DISCUSSION: Phytochemical analysis of the extract and fractions showed the presence of tannins and flavonoids in all of them (**Table 1**). It can be seen that this plant is rich in flavonoids and tannins, and these may be responsible for the observed antibacterial activity. The flavonoids found in some plants have been reported to exhibit antimicrobial activities ²⁵. Also, tannins have been reported to demonstrate antibacterial activity ²⁶.

The TLC separation of the extract and fractions show that each of them contains constituents that have unsaturated/pi bonds and are phenolic in nature (**Tables 2**, and **3**). In addition, some constituents demonstrate antioxidant activity (**Table 4**). From the Rf values of the spots found in the various fractions it cannot be readily ascertained whether it is only the phenolic constituents that are exhibiting antioxidant activity. Rather, the more likely explanation is that the plant contains some other non-phenolic compounds responsible for its recorded antioxidant effect. So, whether these compounds are acting separately or in synergy to give the antibacterial activity reported here remains to be investigated.

Phenolic compounds have variously been reported to show antimicrobial activity ²⁷. The mechanisms of action include adsorption and disruption of

microbial membranes, interaction with enzymes, and metal ion deprivation ²⁸. Phenolic compounds are also reported to have antioxidant effect ^{29, 30}. Some non-phenolic compounds have been reported to show antioxidant activity ³¹, and antimicrobial

effect ³². Therefore it can be inferred that it is the presence of these types of constituents that are responsible for the observed antibacterial activity of this plant, as reported in this work.

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TABLE 1: PHYTOCHEMICAL COMPONENTS OF THE METHANOL EXTRACT, N-HEXANE AND ETHYL ACETATE FRACTIONS OF THE LEAF OF *ERIOSEMA PSORALEOIDES*

Bioactive component test	Methanol extract	n-Hexane fraction	Ethyl acetate fraction
1. Test for Alkaloids:			
(i) (Dragendorff's)	-	-	-
(ii) (Mayer's)	-	-	-
(iii) (Picric Acid)	-	-	-
2. Test for Tannins:			
(i)(Ferric chloride test)	+	+	+
(ii)(Bromine water test)	+	+	+
3. Test for Phlobatannins:			
(Hydrochloric acid test)	-	-	-
4. Test for Flavonoids:			
(Shinoda reduction test)	+	+	+
Test for Anthraquinones			
(Borntrager's test)	-	-	-
6. Test for Saponins:			
(i)(Frothing test)	-	-	-
(ii)(Sodium bicarbonate test)	-	-	-
(iii)Haemolysis test	-	-	-
7. Test for Cyanogenic glycosides	-	-	-
8. Test for Cardiac glycosides:			
(i) (Salkowski test)	-	-	-
(ii) (Keller-Killiani test for deoxy sugars)	-	-	-

Key: (-) absent; (+) present.

TABLE 2: DETECTION OF SPOTS ON TLC PLATES BY IODINE VAPOUR

Extract	R _f Value of Spot	Colour observed	Inference
Methanol extract	0.38	Dark brown	Unsaturated/Pi bond compound present
n-Hexane fraction	0.39	Dark brown	Unsaturated/Pi bond compounds present
	0.57	Dark brown	
	0.66	Dark brown	
Ethyl acetate fraction	0.22	Dark brown	Unsaturated/Pi bond compounds present
	0.36	Dark brown	

Mobile phase = Chloroform: n-Hexane (4:2); Stationary phase = Silica gel 60F254.

TABLE 3: DETECTION OF SPOTS ON TLC PLATES BY FERRIC CHLORIDE SPRAY REAGENT

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Extract	R _f Value of Spot	Colour Observed	Inference		
Methanol extract	0.01	Brown	Phenolic constituent present		
n-Hexane fraction	0.26	Brown	Phenolic constituent present		
	0.60	Brown	Phenolic constituent present		
	0.86	Brown	Phenolic constituent present		
Ethyl acetate fraction	0.06	Brown	Phenolic constituent present		

Mobile phase = Chloroform: n-Hexane (4:2); Stationary phase = Silica gel 60F254.

TABLE 4: DETECTION OF SPOTS ON TLC PLATES BY DPPH SPRAY REAGENT

Extract	R _f Value of Spot	Colour Observed	Inference
Methanol extract	0.26	Bright yellow	Antioxidant constituent present
	0.35	Bright yellow	Antioxidant constituent present
	0.63	Bright yellow	Antioxidant constituent present
n-Hexane fraction	0.07	Bright yellow	Antioxidant constituent present
	0.57	Bright yellow	Antioxidant constituent present
Ethyl acetate fraction	0.06	Absence of bright yellow	Antioxidant absent
	0.07	Absence of bright yellow	Antioxidant absent
	0.19	Absence of bright yellow	Antioxidant absent

Mobile phase = Chloroform: n-Hexane (4:2); Stationary phase = Silica gel 60F254.

TABLE 5: RESULT OF SENSITIVITY TEST OF *ERIOSEMA PSORALEOIDES* EXTRACT AND FRACTIONS AT 10mg/mL EACH, ON THE UNDERLISTED MICRO-ORGANISMS, WITH THE INHIBITION ZONE DIAMETERS (IZD) IN MM

			/				
Extract/	S.A.	S.A.	E.C.	K.A.	P.V.	P.A.	E.C.
Fraction	ATCC		ATCC				
Methanol	10*±0.03	10±0.05	-	-	-	6±0.02	-
n-Hexane	8 ± 0.02	6 ± 0.03	-	-	-	-	-
Ethylacetate	10.5 ± 0.1	7 ± 0.03	-	-	-	-	-
Gentamicin #	6 ± 0.02	-	5 ± 0.01	-	-	-	-
DMSO	-	-	-	-	-	-	-

Key: S.A. (ATCC) = Staphylococcus aureus ATCC 25923, S.A. = Staphylococcus aureus Clinical isolate, E. C. (ATCC) = Escherichia coli ATCC 35219, E.C. = Escherichia coli Clinical isolate, K.A. = Klebsiella aerogenes Clinical isolate, P.V. = Proteus vulgaris Clinical isolate, P.A. = Pseudomonas aeruginosa Clinical isolate, DMSO = Dimethyl sulphuroxide solvent, # = Gentamicin, 4μg/ml, - = No zone of inhibition * = Inhibition Zone Diameter, in (mm), Diameter of cork borer = 6mm

Also, in the sensitivity test conducted for these extracts against the selected bacteria for this study, three of them showed antibacterial activity as they inhibited growth in two of the organisms, *Staphylococcus aureus* ATCC 25923 and *S. aureus* clinical isolate, while the methanol extract showed activity against *Pseudomonas aeruginosa* clinical isolate, in addition (**Table 5**).

In this sensitivity test, 10mg/ml of each of the extract and fractions was tested against all the test micro-organisms viz., *Staphylococcus aureus ATCC 25923, Escherichia coli* ATCC 35219, and the clinical isolates of *Staphylococcus aureus, Escherichia coli, Klebsiella aerogenes, Proteus vulgaris* and *Pseudomonas aeruginosa;* with 4ug/ml of gentamicin(standard drug) and DMSO (solvent) acting as positive and negative controls, respectively. Their activity was compared using inhibition zone diameters (IZD). The greater the IZD (mm), the greater the susceptibility of the organism to the antibacterial effect of the extract/fraction ³³.

Our results show that the IZD of the extract/fractions and standard drug against SA ATCC are in the decreasing order: ethyl acetate fraction, 10.5mm > methanol extract, 10mm > nhexane fraction, 8mm > Gentamicin, 6mm > DMSO, 0mm; against SA clinical isolate, they are in the decreasing order: methanol extract, 10mm > ethyl acetate fraction, 7mm > n-hexane, 6mm; against PA clinical isolate, only the methanol extract at 6mm was active; and against EC ATCC, only Gentamicin at 5mm was active. DMSO did not inhibit the growth of any of the test organisms, hence it can be inferred that it did not contribute to the antibacterial activity of the extracts. From these results, all the plant extract and fractions showed

higher inhibitory activity against these test organisms than the standard drug, Gentamicin. This may suggest that pure compounds isolated from these extracts could be more effective than Gentamicin in inhibiting the growth of these particular test organisms. A pointer to this suggestion could be seen from the fact that SA clinical isolate was found to be insensitive or resistant to the activity of Gentamicin, whereas the plant extract and fractions demonstrated significant activity (p<0.05) against it. This observed resistance could be due to previous exposure of this clinical isolate to Gentamicin. Again, the observed activity of the methanol extract against the SA clinical isolate, and SA ATCC (Gram positive), and PA clinical isolate (Gram negative) suggests that the plant has a broad spectrum of antibacterial activity.

In order to gauge the minimum inhibitory concentration (MIC) of the extract/fractions against the organisms found to be sensitive to them, serial dilutions of the extracts were prepared from 10mg/ml, 8mg/ml, 6mg/ml, 4mg/ml, to 2mg/ml. The logarithm of these concentrations were then plotted on a graph against the square of their IZD for each bacteria earlier found to be susceptible (Fig. 1 to 7). The MIC was then calculated from the antilogarithm of the value on the x-axis where the linear graph cuts it; at this point, the IZD is zero. This value is taken as the MIC. The MIC of the extract and fractions against SA ATCC 25923 in decreasing order are: ethyl acetate fraction, 1.26mg/ml> n-hexane fraction, 1.41mg/ml > methanol extract, 1.78mg/ml; and their MICs against SA clinical isolate in decreasing order are: ethyl acetate fraction, 3.89mg/ml> methanol extract, 3.98mg/ml> n-hexane fraction, 4.47mg/ml; and the MIC of the methanol extract against PA

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clinical isolate is 5.49mg/ml (**Table 6**). From these values, it is evident that the MICs of all the extract/fractions against SA ATCC25923 are significantly (p<0.05) higher than their MICs against SA clinical isolate.

This fact tends to buttress the suggestion that the clinical isolates of this organism may have become more resistant due to prior exposure to antibacterial agents. And, out of the three extracts the most active appears to be the ethyl acetate fraction.

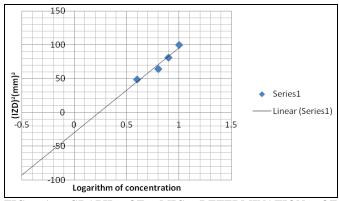


FIG. 1: GRAPH OF MIC DETERMINATION OF METHANOL EXTRACT ON *S. AUREUS*, ATCC **25923** MIC = antilog of intercept on x-axis (0.25) = 1.78mg/ml.

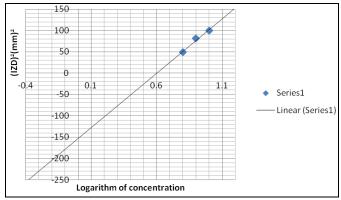


FIG. 2: GRAPH OF MIC DETERMINATION OF METHANOL EXTRACT ON S. AUREUS, CLINICAL ISOLATE MIC = antilog of intercept on x-axis (0.6) = 3.98mg/ml.

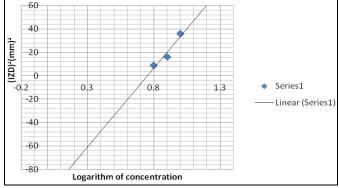


FIG. 3: GRAPH OF MIC DETERMINATION OF METHANOL EXTRACT FOR *P. AERUGINOSA* MIC = antilog of intercept on x-axis (0.74) = 5.49mg/ml.

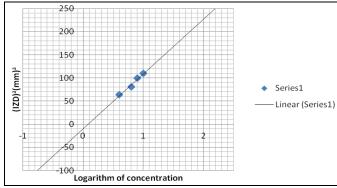


FIG. 4: GRAPH OF MIC DETERMINATION OF ETHYL ACETATE FRACTION FOR *S. AUREUS*, ATCC 25923 MIC = antilog of intercept on x-axis (0.1) = 1.26mg/ml.

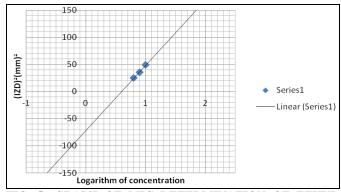


FIG. 5: GRAPH OF MIC DETERMINATION OF ETHYL ACETATE FRACTION FOR S. AUREUS, CLINICAL ISOLATE MIC = antilog of intercept on x-axis (0.59) = 3.89mg/ml.

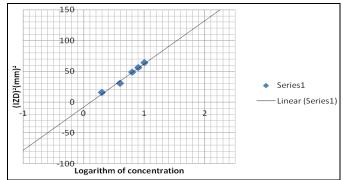


FIG. 6: GRAPH OF MIC DETERMINATION OF N-HEXANE FRACTION FOR S. AUREUS, ATCC 25923 MIC = antilog of intercept on x-axis (0.15) = 1.41 mg/ml.

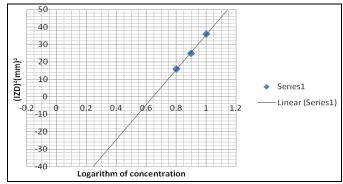


FIG. 7: GRAPH OF MIC DETERMINATION OF N-HEXANE FRACTION FOR S. AUREUS, CLINICAL ISOLATE MIC = antilog of intercept on x-axis (0.65) = 4.47mg/ml.

TABLE 6: SUMMARY OF THE MINIMUM INHIBITORY CONCENTRATIONS (MICS) OF THE METHANOL EXTRACT, N-HEXANE FRACTION AND ETHYL ACETATE FRACTION OF *ERIOSEMA PSORALEOIDES* LEAF ON THE UNDERLISTED MICRO-ORGANISMS

UNDERLISTED MICKO-ORGANISMS					
Extract/ Fraction	S. aureus (ATCC 25923) (mg/ml)	S. aureus Clinical isolate (mg/ml)	P. aeruginosa (mg/ml)		
Methanol extract	1.78	3.98	5.49		
n-Hexane fraction	0.15	4.47	-		
Ethyl acetate fraction	1.26	3.89	-		

CONCLUSION: It is noteworthy that whereas Staphylococcus aureus clinical isolate was found to be resistant to the standard drug, Gentamicin, it was susceptible to the antibacterial activity of the methanol extract, n-hexane and ethyl acetate fractions of the plant Eriosema psoraleoides. This suggests that these extract and fractions may be treating infections in caused Staphylococcus aureus. By providing a scientific evidence of the antibacterial activity of these extracts in vitro, this work appears to validate the use of Eriosema psoraleoides by traditional medicine practitioners to treat wounds and as chewing stick to treat dental caries.

To the best of our knowledge, the antibacterial activity of *Eriosema psoraleoides* is being reported for the first time here since no previous reports have been cited in the literature.

ON-GOING WORK: Further work is on-going for the isolation and characterization of constituents of these extract and fractions in order to obtain the pure active compounds present in them.

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