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In-vitro Antimicrobial Activity of Essential Oils and Different Organic Extracts of *Lippia alba*

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Abstract

The aim of this study was to investigate antibacterial activity of essential oils and different organic extractives extracted from the medicinal plant *Lippia alba*. The chemical composition of the hydrodistilled oil was determined by GC-MS spectroscopy. Typical eighty eight components representing 96.14% of the total oil was identified of which spathulenol (3.03%), caryophyllene oxide (6.39%), aromadendrene oxide-(2) (2.82%), alloaromadendrene oxide-(1) (2.46%), ledene oxide-(ii) (2.8%), α-caryophyllene, (1R)-(-)-myrtenal, 1-octen-3-ol (2.77 at.%), 1H-cyclopenta[1,3] cycloprop[1,2]benzene and myrtenyl acetates (3.29%) were the major components. The antibacterial activity was determined in vitro using agar diffusion method and the MIC determination test against six pathogenic bacteria. The essential oils and different extracts (5.0 µL disc^{-1} correspond to 500 and 250 µg disc^{-1}) displayed a great potential of antibacterial activity against Gram-positive (*Sarcina lutea*, *Bacillus subtilis*) and Gram-negative bacteria (*Xanthomonas campestries*, *Escherichia coli*, *Pseudomonas* sp. and *Klebsiella preumoniae*). The zones of inhibition of different concentrations of the essential oils and the extracts against the tested bacteria were found in the range of 6~23 mm and the minimum inhibitory concentrations (MIC) were recorded between 1.95~500 µg disc^{-1}. The results obtained from this study demonstrated that the natural product derived from *Lippia alba* might be used in pharmaceutical, cosmetic and agro-industries as natural preservatives or flavoring to control pathogenic microbes.

Keywords: *Lippia alba*; Essential oils; Organic extracts; Antibacterial activity; GC-MS spectroscopy

Introduction

Medicinal plants are an important therapeutic aid for various ailments. Different scientific experiments such as biological activity, medicinal and/or antimicrobial properties on the plant components were first documented in the late 19th century [1]. In the light of herbal medication, Bangladesh possesses a rich flora of medicinal plants. Out of 5000 species of phanerogams and pteridophytes, more than a thousand are regarded as having medicinal properties and more than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh [2].

Over the past 20 years, there has been an increased interest in the investigation of natural materials as sources of new antibacterial agents. In the recent years, the development of resistance of pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* etc. against antibiotics has become a difficult issue caused by the indiscriminate use of modern antibiotics [3-5]. Furthermore, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Pneumococcus*, vancomycin-resistant *Enterococcus faecalis* (VRE), and multidrug-resistant *Mycobacterium tuberculosis* (MDRTB) are now commonplace pathogens that are proving difficult to treat effectively [6]. Thus, it is urgent need to find out new antimicrobial agents. This demand has driven scientists to investigate the effectiveness of inhibitory compounds such as essential oils, and extracts from plants. Thus essential oils and plant extracts are promising natural antimicrobial agents with potential applications in food, pharmaceutical and/or agro industries for controlling of various pathogens and spoiling bacteria [7].

Lippia alba (Mill.) N.E. belongs to the Verbenaceae family. It is abundantly present in tropical and subtropical regions including south of the United States of America (Florida), north of Argentina, Australia, India and Bangladesh [8-10]. Leaves are used as an infusion against states of excitement, hypertension, digestive troubles, nausea and cold, to heal wounds sore throat and flu and as syrup against cough and bronchitis. An infusion of the roots is also used against bad colds and coughs. It has also been used as a sedative, against hypertension, flatulence and pain [11], stomach ache [12], diarrhea [13], anaemia [14], skin diseases (macerate is used for washing), headaches (the plant is crushed and used as a poultice). Beside these, this plant also have cytotoxic, antioxidant, anti-inflammatory, neurosedative [15], analgesic, antiprotozoal [16], antiviral, antifungal and antibacterial activities [10].

In this study, we examined the chemical composition of the essential oils extracted from the leaves of *Lippia alba* by GC–MS spectroscopy and tested the antibacterial efficacy of the essential oils and various organic extracts with emphasis for the possible future use of the extracts as an alternative to the chemical bactericides.

Materials and Methods

All the chemicals used in this study were of analytical grade. Ethanol, methanol, chloroform, petroleum ether, petroleum sprit, ethyl acetate, dichloromethane were purchased from Merk, Germany and Fluka. Before use, all the solvents were further purified by distillation.

Healthy, disease free, mature *Lippia alba* leaves were collected from

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the Islamic University, campus, Kustia, Bangladesh in the period of December. A typical image of the plant is shown in Figure 1. The plant was identified and confirmed by Prof. Dr. Oliur Rahman, Department of Botany, Dhaka University, Bangladesh. The air dried leaves (200.0 g) of *Lippia alba* were subjected to hydro distillation chamber for 3 hrs using a clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4°C until further analysis.

In the course of extraction process, the air dried leaves of *Lippia alba* were pulverized into powdered form and then the dried powder (50.0 g) was then extracted with ethanol, methanol, chloroform, petroleum ether, petroleum spirit, ethyl acetate and dichloromethane separately at room temperature for 7 days. The obtained extract is then named as ethanol, methanol, chloroform, petroleum ether, petroleum spirit, ethyl acetate and dichloromethane extracts, respectively. The organic solvents were evaporated by vacuum rotary evaporator at 50°C

The GC-MS spectrum for the essential oil of *Lippia alba* was carried out using total ion monitoring mode on a Varian 3800 gas chromatograph interfaced to a Varian Saturn ion trap 2200 GC-MS spectrometer. The temperatures of the transfer line and ion source were 280°C and 275°C, respectively. Ions were obtained by electron ionization mode. The VF-5 capillary column (30 m length, 0.25 mm I.D. and 0.25 µm film thickness) was used. A 20% split injection mode was selected with a solvent delay time of 3 min with injection volume 0.20 µL. The initial column temperature was started at 50°C for 1 min, programmed at 8°C min^{-1} to 200°C and heated until 280°C at 10°C min^{-1}. Injection port was set at 250°C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Molecular ions (mass range: 40 to 500 mz^{-1}) were monitored for identification. The relative percentage of the oil constituents was expressed as percentage by peak area normalization.

Identification of components of the essential oil was based on their retention indices, relative to a homologous series of n-alkane (C8 to C20) on the VF-5 capillary column under the same operating conditions and computer matching with the GC-MS spectra from the Wiley 6.0 MS data.

The following six microorganisms such as: (i) *Sarcina lutea* (IFO 3232), (ii) *Bacillus subtilis* (IFO 3026), (iii) *Escherichia coli* (IFO 3007), (iv) *Pseudomonas* sp. (ATCC 13867), (v) *Klebsiella pneumoniae* (ATCC 10031) and (vi) *Xanthomonas campestries* (IAM 1671) were used in the antibacterial test. The strains were provided by Prof. Yong Se Lee, Department of Bioresource Technology, College of Agriculture and Environmental Science, Daegu University, Korea. Cultures of the each bacterial strain were maintained on Luria-Bertani (LB) agar medium at 4°C.

Figure 1: Image of the studied medicinal plant *Lippia alba*.

The dried extracts were dissolved in the same solvent used for their extraction and sterilized by filtration using 0.22 µm sterile millipore filter (Millipore Corp., Billerica, MA, USA). The antibacterial test was carried out by the agar disk diffusion method with little modification [17]. The test was done using 100.0 µL of standardized inoculum suspension containing 10^7 CFU mL^{-1} of the bacteria. The essential oil was diluted 1:5 (v/v) with methanol and aliquots of 15.0 µL were spotted onto the sterile Whatman No. 1 filter paper disks (6.0 mm diameter); while 10.0 µL of 30.0 mg mL^{-1} of each organic extract (300 µg $disk^{-1}$) was applied on the filter paper disks and placed on the inoculated LB agar. Negative controls were prepared using the same solvents employed to dissolve the samples. Standard antibiotic, streptomycin (20 µg $disk^{-1}$ from Sigma-Aldrich Co., St. Louis, MO, USA) was used as positive control for the tested bacteria. The plates were incubated micro aerobically at 37°C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition against the tested bacteria. Each assay in this experiment was replicated three times.

Minimum inhibitory concentrations (MICs) of the samples were tested by the standard NCCL method with little modification [18]. Active cultures for MIC determination were prepared by transforming a loopful of cells from stock cultures to flasks and inoculated in LB medium and incubated at 37°C for 24 hrs. The cultures were diluted with fresh LB broth to achieve optical density of 10^7 CFU mL^{-1} for the test organisms at 600 nm by UV/Vis Spectrophotometer (Optizen 2120UV and Optizen III.) Dilutions, to get the final concentrations ranging from 0 to 1000.0 µg.mL^{-1} of essential oil and various organic extracts in Luria-Bertani broth medium, were prepared in 96 well microplates. Finally, 20.0 µL inoculum of each bacterial strain (10^7 CFU mL^{-1}) was inoculated onto the microplates and the tests were performed in a volume of 200.0 µL. The plates were incubated at 37°C for 24 hrs. The lowest concentrations of the test samples, which did not show any visual growth of test organisms after macroscopic evaluation, were determined as MICs, which were expressed in µg mL^{-1}. The MICs were measured for the oils and various extractives, as well as for standards of pure compounds a-humulene, spathuleno and eugenol, which were tested on the same cultures under identical conditions to compare their activity with those of the investigated oil and extracts.

The statistical analysis of the essential oil and the different extracts were assayed for antibacterial activity. Each experiment was run in triplicate, and mean values were calculated. The statistical analysis was carried out employing one way ANOVA ($p<0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

Results and Discussion

The chemical composition of the essential oil of *L. alba* extracted by hydrodistilled process was analyzed by GC-MS spectroscopic method. Typical eighty-eight (88) components representing 96.14% of the total essential oil were identified. The results, components with atomic percentage (%) and possible names are tabularized in (Table 1).

The antibacterial activity of essential oil, different extractives of *Lippia alba* against the employed bacteria were qualitatively assessed by the presence or absence of inhibition zones. The results are shown in (Table 2). The essential oil exhibited antibacterial activity against two Gram-positive and four Gram-negative bacteria at the concentrations of 15.0 µL of 1:5 (v/v) dilution with MeOH. The oil exhibited a potent inhibitory effect against *Pseudomonas* sp., *E. coli*, *K. pneumoniae*, *B.*

No.	Name of compounds	Retention time (Rt)	Composition (at.%)	No.	Name of compounds	Retention time (Rt)	Composition (at.%)
1	1-Octen-3-ol	6.703	2.77	45	Benzene,1,2-dimethoxy-4-(2-propenyl)-	14.235	0.95
2	5-Hepten-2-one,6-methyl-	6.805	2.12	46	Bicyclo[5.2.0]nonane,2-methylene-4,8,8-	14.62	2.39
3	2,6-Dimethyl-1,3,5,7-octatetraene,E,E-	7.598	0.71	47	1,6,10-Dodecatriene,7,11-dimethyl-3-me	14.939	1.08
4	E,Z-3-Ethylidenecyclohexene	7.907	0.55	48	alpha.-Caryophyllene	15.184	2.72
5	N-Carbobenzyloxy-S-benzylcysteine sulf	8.053	0.46	49	1H-Cycloprop[e]azulene,decahydro-1,1,	15.258	0.74
6	1,4-Cyclohexadiene,1-methyl-4-(1-meth	8.162	0.41	50	Naphthalene,1,2,3,4a,5,6,8a-octahydr	15.433	1.22
7	Terpineol, cis-beta.-	8.451	0.47	51	3-Buten-2-one,4-(2,6,6-trimethyl-1-cycl	15.516	0.41
8	1,3-Cyclohexadiene,1-methyl-4-(1-meth	8.162	0.41	52	1H- Cyclopenta[1,3]Cycloprop[1,2] benzene	15.574	5.01
9	1,3-Cyclohexadiene,-1-carboxaldehyde,2	8.542	0.41	53	Ethanone,1-(1a,2,3,5,6a,6b-hexahydro-3	15.948	0.4
10	3,5-Heptadienal,2-ethylidene-6-methyl-	8.784	0.6	54	Naphthalene,1,2,3,4a,5,6,8a-octahydr	16.096	1.76
11	1,6-Octadien-3-ol,3,7-dimethyl-,2-amin	8.936	2.36	55	Tricyclo[5.2.2.0(1,6)]undecan-3-ol,2-me	16.203	0.4
12	2-Cyclohexen-1-ol, 4-ethyl-1,4,dimethyl-	9.041	0.4	56	Aromadendrene oxide -(2)	16.265	2.82
13	Phenylethyl Alcohol	9.353	0.41	57	1,6,10-Dodecatriene-3-ol,3,7,11-trimethy	16.635	2.3
14	3,5-Heptadien-2-ol,2,6-dimethyl-	9.454	0.44	58	1-Hydroxy-11,7-dimethyl-4-isopropyl-2,7-c	17.043	0.68
15	2,6,10-Dodecatrien-1-ol,3,7,11-trimethy	9.747	2.22	59	Caryophyllene oxide	17.175	6.39
16	1,5,7-Octatrien-3-ol,2,6-dimethyl-	10.007	0.36	60	Cyclohexene,6-(2butenyl)-1,5,5-trimeth	17.315	0.28
17	Biclo[3.1.1]hept-3-en-2-ol,4,6,6-trim	10.425	2.44	61	beta.-Guaiene	17.488	0.32
18	Cyclohexanone,2-(2-nitro-2-Propenyl)-	10.551	0.69	62	Cubenol	17.567	0.57
19	3-Cyclohexene-1-one,3,5,5-trimethyl-	10.63	0.36	63	2-Butenal,2-methyl-4-(2,6,6-trimethyl-1	17.681	0.53
20	(1R)-(-)-Myrtenal	10.852	4.71	64	Acetic acid,3-hydroxy-6-isopropenyl-4,8	17.907	0.42
21	o-Mentha-1(7),8-dien-3-ol	11.014	0.48	65	1H-3a,7-Methanoazulene-6-methanol,2,	18.11	1.84
22	5-Isopropenyl-2-methylcyclopent-enec	11.11	0.53	66	1R,4S,7S,11R-2,2,4,8-Tetramethyltricycl	18.179	0.58
23	2-Cyclohexen-1-ol,2-methyl-5-(1-methyl	11.218	0.93	67	2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)	18.302	2.33
24	3,6-Octadien-1-ol,3,7-dimethyl-,(Z)-	11.366	0.57	68	Tricyclo[5.2.2.0(1,6)]undecan-3-ol,2-me	18.404	0.92
25	Benzaldehyde,4-(1-methylethyl)-	11.707	2.34	69	Limonen-6-ol,pivalate	18.481	0.49
26	2-Cyclohexen-1-ol,3,7-dimethyl-,(Z)-	11.366	0.57	70	1-Oxapiro[2.5]octane,5,5-dimethyl-4-(3	18.549	0.39
27	Benzaldehyde,4-(1-methyl)-	11.707	2.34	71	Ledene oxide-(ii)	18.62	2.8
28	2-Cyclohexen-1-one,3-methyl-6-(meth-	12.209	0.44	72	6-Isopropenyl-4,8a-dimethyl-1,23,, 5,6,7,	19.186	1.23
29	Acetic acid,1,7,7-trimethyl-bicyclo[2.2	12.328	0.4	73	Alloaromadendrene oxide-(1)	19.537	2.46
30	2-Caren-10-al	12.484	0.52	74	Isoaromadendrene epoxide	19.804	0.65
31	1,3-Cyclohexen-1-methanol,4(1-methyle	12.658	0.37	75	1-Heptatriacotanol	19.857	0.34
32	7-Oxabicyclo[4.1.0]hepten,1-methyl-4-	12.749	0.43	76	Aromadendrene oxide-(1)	20.11	0.35
33	2,6,10,10-Tetramethyl-1-oxa-spiro[4.5]de	12.866	0.37	77	Tricyclo[20.8.0.0(7,16)]tricontane,1(2	20.334	0.48
34	(-)Myrtenyl acetate	12.968	3.29	78	2-Pentadecanone,6,10,14-trimethyl-	20.475	0.42
35	gamma.-Elemene	13.089	0.42	79	3,4,4-trimethyl-3-(3-oxo-but-1-enyl)-bic	20.604	0.32
36	2,7-Dimethyl-2,7-octanediol	13.204	0.38	80	2-Butenal,2-methyl-4-(2,6,6-trimethyl-1	20.728	0.36
37	alpha.-Cubebene	13.331	0.49	81	2-Naphthalenol,2,3,4,4a,5,6,7-octahydro	21.232	0.28
38	2,6-Octadien-1-ol,3,7-dimethyl-,acetat	13.445	0.44	82	(-)Spathulenol	21.858	3.03
39	Phenyl,2-methoxy-3-(2-propenyl)-	13.521	0.66	83	Murolan-3,9(11)-diene-10-peroxy	22.037	0.4
40	2,6-Octadien-1-ol,3,7-dimethyl-,acetat	13.771	2.4	84	1,2-Benzenedicarboxylic acid,butyl 2-me	22.692	0.27
41	Capene	13.849	0.57	85	Tetracontane,3,5,24-trimethyl-	23.298	0.33
42	Cyclohexene,1-ethynyl-1-methyl-2,4-bis(	13.932	0.55	86	Phenol,2-(1,1-dimethylethyl)-4-(1-methy	24.424	0.34
43	Naphthalene,1,2,3,5,6,7,8,8a-octahydro-	14.049	1.87	87	2H,8H-Benzo[1,2-b:5,4-b']dipyran-10-pro	44.064	0.82
44	Bicyclo[3.1.1]hept-2-en-6-one,2,7,7trim	14.169	0.44	88	Acetic acid,7,7,10a,12a-tetramethyl-2,5	44.192	1.55
Total components=96.14%							

Table 1: Chemical composition of the essential oil obtained from the GC-MS spectrum of the medicinal plant *Lippia alba*.

subtilis, *X. campestries* and *S. lutea*, with diameter of inhibition zones ranging from of 9.0 to 22.0 mm. Also ethanol, methanol, chloroform, petroleum ether, petroleum spirit, ethyl acetate and dichloromethane extracts of *L. alba* revealed a great potential of antibacterial activity against all the bacteria at the concentrations of 500.0 μg disk^{-1}. Methanol and petroleum spirit extracts showed the strongest antibacterial effect against *S. lutea*, *B. subtilis*, *E. coli* and *X. campestries* with the inhibition zones of 22.0 to 9.0 mm. On the other hand, ethanol, ethyl acetate and dichloromethane extract showed moderate to high antibacterial effects against most of the bacteria that have being tested with the inhibition zones of 9.0 to 15.0 mm. Chloroform and petroleum ether fraction displayed a moderate inhibitory effect against most of the bacteria.

In this study, the oil exhibited half antibacterial activity of nalidixic acid against Gram-positive bacteria. The blind control did not inhibit the growth of the bacteria tested. Methanol and petroleum spirit, extracts showed higher activity compared to that of the ethanol, chloroform, petroleum ether, ethyl acetate and dichloromethane extracts.

The minimum inhibitory concentration (MIC), as shown in (Table 3), for the oils were found to be lower for *B. subtilis* and *Pseudomonas*

Pathogens	Zone of inhibition (mm)										
	EtOH	MeOH	$CHCl_3$	PE	PS	Et-Ac	CH_2Cl_2	EO	NA-30	S	E
Bacillus subtilis	10	18	6	5	12	10	12	12	27	6	32
Sarcina lutea	10	16	6	8	22	12	12	9	30	6	15
Escherichia coli	12	13	8	7	12	11	10	15	28	8	22
Pseudomonas sp.	11	19	7	7	-	10	-	23	27	7	17
Klebsiella preumoniae	13	14	9	9	9	11	15	15	29	7	20
Xanthomonas campestries	14	14	8	6	14	12	9	10	27	6	24

EtOH=Ethanol, MeOH=Methanol, $CHCl_3$=Chloroform, PE=Petroleum ether, PS=petroleum spirit, EtAc= Ethyl-acetate, CH_2Cl_2=Dichloromethane, EO=Essential oil, NA-30=Nalidixic acid (30 µg $disc^{-1}$), S=Spathulenol, E=Eugenol.

Table 2: Antibacterial activity of essential oils, essential components and different extractives of *Lippia alba*. 500.0 µg of extracts or essentials oil were applied for experiment per disc.

Pathogens	Zone of inhibition (mm)							
	EtOH	$CHCl_3$	PE	PS	Et-Ac	EO	S	E
Bacillus subtilis	31.25	250	500	62.5	3.9	15.62	62.5	15.62
Sarcina lutea	31.25	125	125	15.62	7.81	62.5	31.25	15.62
Escherichia coli	15.62	125	125	15.62	1.95	31.25	15.62	31.25
Pseudomonas sp.	31.25	31.25	62.5	-	7.81	15.62	3.9	7.81
Klebsiella preumoniae	15.62	125	62.5	15.62	1.95	31.25	7.81	15.62
Xanthomonas campestries	15.62	62.5	125	15.62	1.95	31.25	31.25	31.25

EtOH=Ethanol, $CHCl_3$=Chloroform, PE=Petroleum ether, PS=petroleum spirit, EtAc=Ethyl acetate, EO=Essential Oil, S=Spathulenol, E=Eugenol.

Table 3: Minimum inhibitory concentration (MIC) values of different extracts, essential oils, essential components of *Lippia alba*. 500.0 µg extracts or essential oil components were applied per disc.

sp. (15.62 µg mL^{-1}). The *E. coli*, *K. pneumoniae* and *X. campestries* (31.25 µg mL^{-1}) have moderate MIC value but it is higher for S. lutea (62.5 µg mL^{-1}). On the other hand, MIC values of the various extracts against the tested bacteria were found to be in the range of 1.95 to 500 µg mL^{-1} (Table 3). The Gram-negative bacteria were found to be more susceptible to the essential oil and various solvent extracts than Gram-positive bacteria. Standard pure compounds such as spathulenol and eugenol were also exhibited potent activity as compared to that of the oils or extractives.

In this present study, the essential oils and various extracts of *L. alba* exhibited potential activity against reference bacterial strains. This activity could be attributed to the essential oils of *L. alba* and probably related to the high content of oxygen containing monoterpenes, represented mainly by aldehydes and alcohols, such as neral/geranial and nerol/geraniol [19]. In our opinion, major components of the oils, caryophyllene oxide (6.39%), 1H-cyclopenta[1,3]cycloprop[1,2] benzene (5.01%), (1R)-(-)-Myrtenal (4.71%), spathulenol (3.03%) and eugenol have key roles for their antibacterial activities. The antibacterial activities of these compounds have been reported by the others groups [7,20]. Pure compounds such as spathulenol and eugenol showed potent antibacterial activity. Also, the antibacterial activity of individual components of essential oils such as spathulenol and eugenol has been reported previously [7,21]. On the other hand, the components in lower amount such as terpineol, benzaldehydes, elemene, caryophyllene, tricyclo[5.2.2.0(1,6)]undecan-3-ol and cubenol also contributed to the antibacterial activity of the oils [22-27]. It is also possible that the minor components might be involved in some type of synergism with the other active compounds [28]. Furthermore, the antibacterial activity of organic extract could be attributed to the presence of some bioactive phytochemicals such as alkaloids, flavonoids, steroids, tannins, phenolic compounds, diterpenoids, etc. in the *L. alba* and these findings are in agreement with the previous reports [19,29-33].

Conclusion

The results of this study are promising and show a possible therapeutic alternative to treat various infections caused by pathogenic bacteria. The findings also support the traditional usage of the *L. alba* plants and suggests that some of the extracts possess compounds with high antibacterial properties. Therefore, new researches have been proposed in order to elucidate the possible action mechanisms involved and to find new bioactive compounds in *L. alba* in the upcoming near future study.

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