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ANTIBACTERIAL ACTIVITIES OF CRUDE EXTRACT OF *CROTON MACROSTACHYUS* LEAVES AND PURE COMPOUND (METHYL LAURATE) ISOLATED FROM IT

Geremew Tafesse*[1] and Abreham Assefa[1]

[1] *Department of Biology, Dilla University, Dilla, Ethiopia*
Email: abrehamas@du.edu.et/abrishasf@mail.com (A.A)
* *Corresponding author; Email: geremewtaf@gmail.com*

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Abstract

Croton macrostachyus Delil belongs to one of the largest genera of the family Euphorbiaceae, called Croton under the subfamily Crotonoideae. The genus Croton is ecologically prominent, and an important source of secondary metabolites with antimicrobial properties in tropics and subtropics. The objective of this study was to test the antibacterial property of the leaf extract of Croton macrostachyus and a lauric acid derivative, Methyl Laurate, isolated from it. Crude extract was obtained through phytochemical screening using the solvent acetone. The pure compound Methyl Laurate was isolated by a combined application of column chromatography, gel filtration using Sephadex LH-20 and preparative thin layer chromatography (prep-TLC) following crude extraction. Disk diffusion method was employed to assess antibacterial activities of both the crude and the pure compound on four bacterial strains viz Staphylococcus aureus, Escherichia coli, Salmonella typhi and Shigella boydii. MIC values were also determined for each. NMR data has confirmed that the isolated compound is a lauric acid derivative called methyl laurate. The crude extract, Croton Ethyl-acetate Extract (CEaE) showed strong antibacterial activities against Staphylococcus aureus and Shigella boydii with an MIC value of 6.25 mg/ml. However, the isolated compound, methyl laurate showed strong activities against all tested bacterial species with an MIC of 0.156 mg/ml (156 µl/ml) for Staphylococcus aureus, Salmonella typhi and Shigella boydii while 0.312 mg/ml (312 µg/ml) for Escherichia coli. Results suggest that this plant contains phytochemicals that can combat pathogenic bacteria that might be the rationale for its traditional use in the external wound healing process.

Keywords: Antibacterial, *Croton macrostachyus*, Disk diffusion, Methyl Laurate , MIC

1 Introduction

Croton macrostachyus Delil belongs to one of the largest genera of the family Euphorbiaceae, called Croton under the subfamily Crotonoideae. *Croton* is an ecologically prominent and important source of secondary metabolites with antimicrobial properties in the tropics and subtropics. *C. macrostachyus* is reported to have ethnomedical uses concerning reproductive biology such as stopping bleeding in childbirth, inducing abortion, and serving as a purgative.

The genus *Croton* is used for treatment of several human health problems including diabetes, malaria, dysentery, stomachache, ascariasis, and taeniasis in different areas (Kasa, 1991; Giday *et al*., 2007; Mesfin *et a*l., 2009). Abdominal pain, gonorrhea, wounds, ringworm infestation, hemorrhoids, venereal diseases, cough, rheumatism and as a purga-

tive in cases of ascariasis are also among diseases traditionally treated by different species of *Croton* (Abebe, 1986; Mazzanti *et al*., 1987; Yirga *et al*., 2011). There are also reports for the analgesic, anti-inflammatory, mitogenic, molluscicidal, and larvicidal activities of extracts from different species of *Croton* (Tachibana *et al*., 1993; Karunamoorthi and Ilango, 2010).

Different reports have been made available for some compounds isolated from Genus *Croton* for their *in vitro* antimicrobial activities. Antimicrobial compounds isolated from Croton include flavonoids, alkaloids, and terpenes (Junior *et al*., 2011). Sesquiterpene oxide obtained from the bark *C. stellulifer* has been reported to possess antimicrobial property against some bacterial strains such as *Escherichia coli, Staphylococcus aureus, S. epidermidis, Proteus vulgaris*, and fungal species *Candida albicans* and *Aspergillus fumigatus* (Martins *et al*., 2000).

Bioactive compounds isolated from genus *Croton* include terpenes/terpenoids (monoterpenes, sesquiterpenes, diterpenes and triterpens), alkaloids and flavonoids. For instance, lupeol, a triterpene, is one of a bioactive compound isolated from the genus Croton. Other bioactive compounds such as crotin (a chalcone), crotepoxide (a cyclohexane diepoxide), fatty acids and saponins are also reported from *Croton* (Salatino *et al*., 2007; Schmelzer and Gurib-Fakim, 2008). In Ethiopia, *C. macrostachyus* has folk medicinal uses as purgative and vermifuge, treatment of various skin infections, management of helminthes and venereal diseases and induce abortion (Abate, 1989 in Giday *et al*., 2007; Schmelzer and Gurib-Fakim, 2008). The objective of this study was to test the antibacterial property of the leaf extract of *C. macrostachyus* and a lauric acid derivative, *Methyl Laurate*, isolated from it.

2 Materials and Methods

2.1 Plant Materials Collection

Fresh leaves of *C. macrostachyus* were collected from Dilla, Gedeo Zone, 360 km from Addis Ababa, Ethiopia, in November 2013 at altitude of about 1550 m a.s.l and then identified by the help of botanists. Sample specimens were kept in the Herbarium of Addis Ababa University under voucher number GT 006/2013. Leaves were cleaned of any external contaminants and then dried under shade for about 15 days with a careful and continuous follow up to avoid any contamination. Leaves were then grounded using a general purpose blender to an appropriate size for extraction with the help of mesh (0.5 mm).

2.2 Crude Extraction

The subsequent extraction method was employed to get crude extracts from the plant sample using four different solvents with increasing polarity according to the methods in Rimando et al. (2001) and Sigh, (2008). About 1 kg of *C. macrostachyus* leaves powder was macerated for 24 hours in n-hexane with the ratio of 1:7 and 1:5 (w/v) respectively. After 24 hours filtration was made using a double layer filter paper (Whatmann No. 1) giving filtrates and residues. Residues were then macerated in ethyl acetate with a similar ratio as of n-hexane for another 24 hours. These processes were repeated using ethanol and methanol for two subsequent days (24 hours each). All filtrates were concentrated using Rota-vapor to obtain crude extracts and named as shown in figure 1.

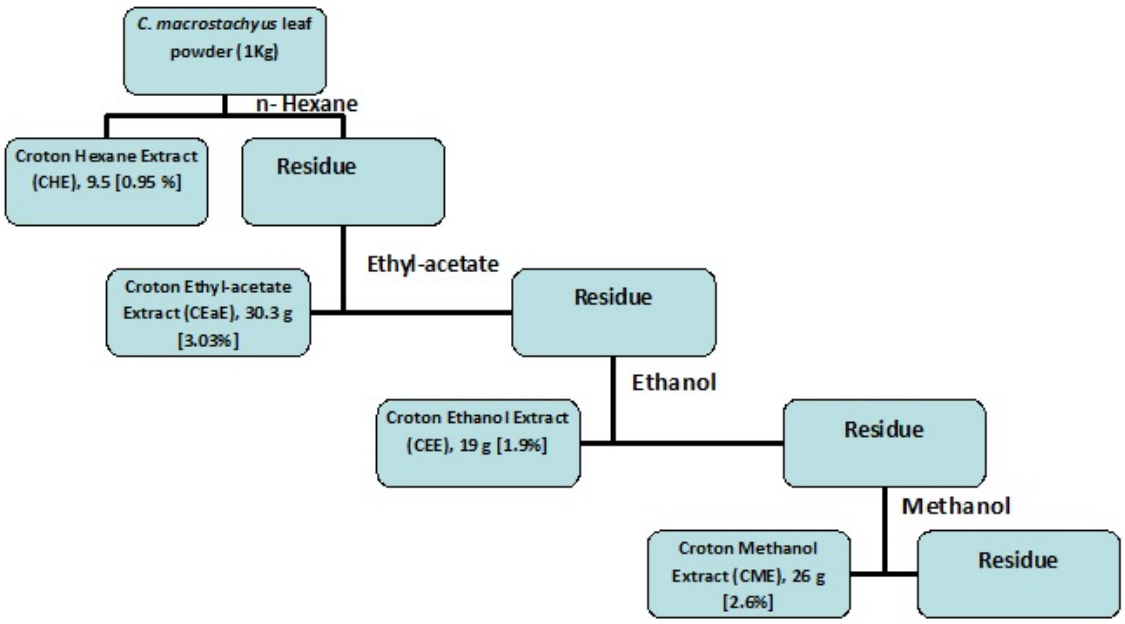

Fig. 1 Flow chart of crude extraction of the leaves of *C. macrostachyus*.

Preliminary antibacterial tests were made for each crude extract to select the most active ones. Based on the preliminary test CEaE (Croton Ethyl-acetate Extract) was selected and then subjected to fractionation according to methods in Shahverdi *et al*. (2005) and Erasto *et al*. (2006) with slight modifications. Fractionation was done for each using flash column chromatography that was packed with silica gel 60 F_{254}. Samples were adsorbed on silica gel 100 (1:2) and then applied to the column followed by the addition of solvents with increasing polarity (100% n-hexane to 100% chloroform then to 100% ethyl acetate ending with methanol:ethyl acetate [1:3]). Fractions of similar profiles on the TLC were combined together and prep-TLC was made for each using an appropriate solvent system to get bands. Prep-TLC was examined under UV light of 254/365 nm wavelength and then each band was carefully cut out and dissolved separately in chloroform and then filtered using a 9 mm Whatmann NO 1 filter paper to separate from the silica gel. The obtained filtrate was left to dry until the solvent completely evaporates, which was then weighed and subjected to NMR analysis (Rimando *et al*., 2001).

2.3 Antibacterial Susceptibility Test

Four bacterial species selected on the bases of their pathogenicity to cause frequent and series infections in human were used. Standard bacterial strains *S. aureus* (ATCC25223), *S. typhi* (ATCC13311), *Escherichia coli* (ATCC23923) and *S. boydii* (ATCC9207) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia.

The disk diffusion method was employed for the antibacterial sensitivity tests according to methods of Onyeagba *et al*. (2004) and Taiwo *et al*. (2007) with some modification. The absorbent filter paper was used to prepare disks with a diameter of 6 mm each. The paper disks were dispensed in batches in a screwed capped bottle and sterilized at 160 ^{o}C for an hour. The four bacterial strains were made to grow and activated on their selective media: S.S agar for *S. typhi* and *S. boydii*, Malliton-Salt agar for *S. aureus* and Mackonkey agar for *Escherichia coli*. These plates were incubated at 37 ^{o}C for 24 hours.

Few colonies of each strain were transferred with a sterile inoculating loop to a liquid medium (nutrient broth) until turbidity was adjusted to that of McFar-

land 0.5 turbidity standard. Four plates containing Muller-Hinton agar were prepared where the four bacterial strains were streaked using sterile cotton swabs (Tadeg *et al*. 2005; Taiwo *et al*., 2007). The crude extract (CEaE) was dissolved in 3% Tween 80 at concentrations of 50 mg/ml whereas the pure compound, methyl laurate was dissolved at 1 mg/ml in the same solvent. Four disks were impregnated with 30 μ of the crude extract and another 4 with 30 μL of the pure compound and all left to dry. Four plates each with a specific test bacterium were divided into four quadrants. On the first quadrant a disk impregnated with crude extract and on the second a disk impregnated with the pure compound was kept. A stock solution of standard antibiotics (CAF for *S. typhi*; ERY for *S. aureus*; AMP for *E. coli* and CIP for *S. boydii*) were prepared at a concentration of 2.5mg/ml each). A 30 μL of each was loaded on a disk and kept on the 3rd quadrant to serve as a positive control, and a disk immersed in 1ml of 3% Tween 80 was kept on the rest serving as a negative control. All plates were then incubated at 37 ^{o}C for 24 hours after which zone of inhibition was measured. All tests were conducted in triplicate to confirm results.

2.4 Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) of the crude extract (CEaE) and the pure compound (methyl laurate) was determined according to methods in Adebolu and Oladimeji (2005) and Doughari *et al*. (2008). The disk diffusion method was employed as in the susceptibility tests except that disks were impregnated with 50 μL of each prepared concentration of the samples. The crude extract was tested at concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml and 1.56 mg/ml. The MIC value of methyl laurate was tested at concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and 0.03125 mg/ml.

2.5 Data Presentation and Analysis

1D spectrum (^{1}H NMR and ^{13}C NMR) was used to get the number of proton and carbon respectively while 2D spectrum (DEPT, HMBC, HMQC, and COSY) to confirm and plot results for each compound. The structure of each compound was identified based on available literature and constructed using Chem-Draw® software. Results of antibacterial tests were recorded by measuring (in mm) zones of growth inhibition by the controls. The presence/absence of growth was recorded for MIC values. The average (Mean $\pm$ SEM) value of three tests was taken for all results. One way ANOVA (Tukey) was used to compare results with 95% confidence intervals where P-value less than 0.05 showing significant difference.

3 Results and Discussions

3.1 Isolated Compound

The result of 1D spectrum (^{1}H NMR and ^{13}C NMR) and 2D spectrum (DEPT, HMBC, HMQC, and COSY) had confirmed that the pure compound isolated from the crude extract (CEaE) was a 13-C compound, with a formula of $C_{13}H_{26}O_2$ and Mol. Wt. 214.3 (Riháková *et al*., 2001). It is a lauric acid derivative named as methyl laurate or methyl dodecanoate (Figure 2). Although other compounds were also been isolated methyl laurate has been selected for the fact that it had shown strong antibacterial activities at a concentration of 16 mg/ml with the MIC value of 0.3 mg/ml as reported by the same author (data not shown).

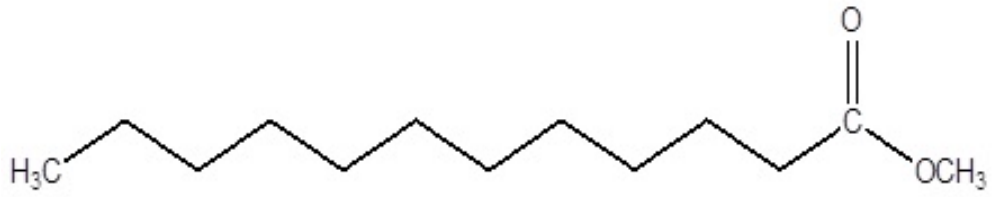

Fig. 2 The proposed structure of methyl laurate isolated from *C. macrostachyus* leaf extract (CEaE).

3.2 Antibacterial Activities

The ethyl acetate crude extract of *C. macrostachyus* leaves (CEaE) showed antibacterial activities against all tested bacterial strains at the concentrations of 50 mg/ml. The mean zones of growth inhibitions of CEaE were 23.67±0.6, 19.3±1.6, 20±1.0, and 26.6±1.1 against *S. aureus, E. coli, S. typhi* and *S. boydii* respectively (Table 1). As shown in Table 1, the mean zones of growth inhibitions recorded by the isolated compound, methyl laurate were 25.67±1.2, 22±1.0, 25.5±0.58 and 26±2.0 for *S. aureus, E. coli, S. typhi* and *S. boydii* respectively. Though significant difference to the negative control (P = 0.00), both the crude extract and methyl laurate have antibacterial activities against all tested bacteria without significant differences with the respective standard drug (P > 0.05)

Table 1 Growth inhibition (Mean ± SEM) of the four bacterial strains by crude ethyl acetate extract (CEaE) of *C. macrostachyus* leaves and the isolated compound, Methyl laurate.

Test Material	Conc.	*S. aureus*		*E. coli*		*S. typhi*		*S. boydii*	
		Mean± SEM	P- value	Mean± SEM	P- value	Mean± SEM	P- value	Mean± SEM	P- value
Tween 80	1ml	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00	0±0.0	0.00
Drug*	2.5mg/ml	27±1.3		24±1.0		28±1.0		28±1.0	
CEaE	50mg/ml	23.67±0.6	0.39	19.3±1.6	0.01	20±1.0	0.00	26.6±1.1	0.61
Methyl laurate	1mg/ml	25.67±1.2	0.40	22±1.0	0.85	25.5±0.58	0.10	26±2.0	0.43

*Drugs Used: Erytromycin (for *S. aureus*), Ampicilin (for *E. coli*), Chloramphenicol (for *S. typhi*) and Ciproflaxin (for *S. boydii*)

3.3 Minimum Inhibitory Concentration

The MIC of this extract was 6.25 mg/ml to all bacterial strains tested (Table 2). The isolated compound, methyl laurate has shown similar MIC values (0.156 mg/ml) for all tested bacterial strains except *E. coli* that was 0.312 mg/ml (Table 3)

The finding of the present study is in agreement with many other works. Crude extracts obtained from the leaves and stem of *C. macrostachyus* had been reported to show effective inhibitory activities against both Gram negative and Gram positive bacteria (Taniguchi and Kubo, 1993). The crude hydro-alcoholic extract of *C. campestris* leaf has been reported to show antibacterial activities on *S. aureus* and *E. coli* (Junior *et al*., 2011). Martins *et al*. (2000) had reported that essential oil from *C. stellulifer* have growth inhibitory activities against *E. coli*, *S. aureus*, *S. epidermidis* and *Streptococcus faecalis*. The crude methanol extract from *C. pullei* has also shown inhibitory activity of *S. aureus* (Peixoto *et al*., 2013). In the present study, the crude extract of *C. macrostachyus* leaves has the least activity against *E. coli* which can be supported by Panda *et al*. (2010a) that had reported similar results for the aqueous and alcoholic extracts of *C. roxburghii* having a higher antibacterial against *S. aureus* than *E. coli*. The present work, therefore, convinces that this plant has antibacterial phytochemicals.

In the present study methyl laurate showed the best antibacterial activities in this study as other lauric

acid derivatives such as lauric acid carbohydrate esters do in the previous study (Nobmann *et al*., 2009). In addition, many other lauric acid derivatives such as D-laurate A, T-laurate and lauro-sucrose have been reported for their effective antimicrobial activities (Riháková *et al*., 2001). Moreover, lauric acid derivatives such as methyl caprate and methyl laurate have been reported to involve in the manufacturing of detergents and surfactants (Thompson *et al*., 1990; Cermak and Isbel, 2004) due to their ability to fight microbes. These could support the positive result of antibacterial activity observed for methyl laurate in the present work, which might go with its ability to treat such diseases and asserts the traditional use of this plant for skin infection.

Table 2 Minimum Inhibitory Concentration (MIC) of crude ethyl acetate extract (CEaE) of *C. macrostachyus* leaves.

Test Bacteria	Activity					
	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.563 mg/ml
S. typhi	++	+	+	+	-	-
S. boydii	+++	++	+	+	-	-
S. aureus	+++	++	+	+	-	-
E. coli	++	+	+	+	-	-

Table 3 Minimum Inhibitory Concentration (MIC) of methyl laurate isolated *C. macrostachyus* leaves.

Test Bacteria	Activity							
	1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.125 mg/ml	0.625 mg/ml	0.312 mg/ml	0.156 mg/ml	0.078 mg/ml
S. typhi	+++	++	++	++	+	+	+	-
S. boydii	+++	++	++	++	+	+	-+	-
S. aureus	+++	+++	+++	++	+	+	-+	-
E. coli	+++	+++	++	+	+	+	-	-

4 Conclusion and Recommendations

People, especially those living in developing nations like Ethiopia have used and are still using medicinal plants designing their own methods of applications. Scientific proof is, then, crucial to validate the traditional application of such plants along with suggesting the possibility of developing drugs from them. In Ethiopia, this plant is used to heal wounds and get rid of intestinal worms in different rural and suburban areas. The findings of the present study might give an insight that this plant contains secondary metabolites that could combat infectious agents like bacteria. Since the present study focused only on the antibacterial activities of leaf extract and active compound similar studies need to be performed on other parts of the plant. Great effort and attention should be given to conserving this plant by confirming and educating people about its medicinal values. It is also recommendable to check the toxicity profile of the plant using other animal models along with in-vivo tests for drug development from this plant.

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Conflict of interest

The author declare that no conflict of interest.

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