International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.09 pp 247-253, 2016

Characterization of Fraction of *Carica papaya* L. Leaves Ethyl Acetate Extract to African Catfish *Clarias gariepinus* Leucocytes Using UV-Vis, FTIR and GC-MS Methods

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Abstract : The active component in the leaves of *C. papaya* L. such as alkaloids including carpain and pseudocarpain, enzyme (papain, chymopapain, cystatin), tocopherol, amino acids, flavonoids, tannins, nicotine acid, saponins and other phenolic compounds, has the effect of immunomodulatory. Blood cells, especially leukocytes, present important phagocytic function on the immune system regulation. The aim of this study to determine the characterization of a fraction of *Carica papaya* L. leaves ethyl acetate extract to African catfish (*C. gariepinus*) leucocytes using UV-Vis, FTIR and GC-MS methods. Our analytical attention was focused on secondary metabolites which never been reported in the literature. *C. papaya* L. leaves fraction 9 contains active compounds hexanedioic acid, bis(2-ethylhexil) ester and hexanedioic acid, dioctyl ester that are triterpenoid derivatives, were assumed increasing leucocytes number of African catfish. Besides, it has beneficial effects on fish health and enhance the immune system (leucocytes number) and hence they could play an important role in preventing disease outbreaks in aquaculture systems.
Keywords: characterization, fraction of *Carica papaya* L., leucocyte.

Introduction

Papaya (*Carica papaya* L.) is a plant originated from southern Mexico and northern parts of South America. This plant spreads to the continent of Africa and Asia and the Indian state. From India, the plant is spread to many tropical countries, including Indonesia back in the 17th century. Papaya plant is one of the vascular plants that produce seeds, has flowers and is dashed two (dicotile)[1].

C. papaya L. leaves contain alkaloids including carpain and pseudocarpain, enzyme (papain, chymopapain, cystatin), tocopherol, amino acids, flavonoids, tannins, nicotine acid, saponins and other phenolic compounds. They also contain phenol acid (caffeine acid, p-coumarin acid, and acid protocatechuic) as the primary phytochemicals. Chlorogenic acid is found in small amounts, compared with flavonoids and coumarin. Also contain glycosides, carposide, sucrose, dextrose and levulose, dehydrocarpain I and II, choline, vitamin C and E[2,3,4].

The active component in the leaves of *C. papaya* L. has been widely known as antiprotozoal and antidiarrheal, are detoxification capable of neutralizing toxins in the body, as anti-tumor and has the effect of

immunomodulatory[5]. Blood cells, especially leukocytes, present important phagocytic function on the immune system regulation. Leukocytes are also the main producers of lysozyme, the important bacteriolytic agent found in marine and freshwater fish species. Immunostimulants are usually identified by their ability to activate leukocytes in in-vitro tests[6]. African catfish (*Clarias gariepinus*), a commercially important, air-breathing catfish, due to its preferential habitat in a derelict, shallow, and swampy water was cultured in intensive fish culture. This type of practice creates highly stressful environment for fish that further suppresses the immune response and outbreak of infection occur[7]. However, none has been published on the chemical characterization of *C. papaya* fraction affected the leucocytes of African catfish.

Gas chromatography–mass spectrometry (GC–MS) was selected as the method of chemical analysis to identify and quantify the metabolites in leaf extracts obtained using liquid–liquid extraction (LLE). This extraction method made it possible to clean up the extracted sample before derivatization reaction and chromatographic separation and identification. Such a method is extremely useful for the purification of the samples resulting from the interferences due to the biological matrix[8].

The aim of this study to determine the characterization of a fraction of *Carica papaya* L. leaves ethyl acetate extract to African catfish (*C. gariepinus*) leucocytes using UV-Vis, FTIR and GC-MS methods. Our analytical attention is focused on secondary metabolites which have never been reported in the literature.

Material and Methods

Preparation of Carica papaya L Fraction

Healthy, fresh, young and green *C. papaya* L leaves were collected from papaya plants farm in Malang - Indonesia and have been determined in Laboratory of Taxonomy, Department of Biology, Mathematics and Science Faculty of Brawijaya University in Malang – Indonesia. The leaves were air-dried under shade at room temperature for 8 days. They were washed and the stems were removed before use. Then, they were cut into small pieces and blended without adding water or other liquids.

C. papaya L. leaves extract was prepared according to maceration method as described by[9,10]. 100 g *C. papaya* L. blended leaves was soaked in 300 ml ethyl acetate for extraction in a rotary shaker for 48 hours at room temperature. The solution was separated using filter vacuum and filtrate was evaporated by rotary evaporator on 45^{o}C. Finally, the volume of the extract was measured and the extracts were stored at 4 °C until use.

Phytochemical identification of the extracts was determined, such as alkaloid, flavonoid, saponin, tannin, and triterpenoid, as described by[11,12]. Fractionation of *C. papaya* L. leaves extract was determined using chromatography methods.

To determine the eluent of Column Chromatography (CC) were used Thin Layer Chromatography (TLC) method with eluent ratio was methanol:ethyl acetate:N-hexane (1:2:10) as described by[11]. The 0.1 g ethyl acetate extracts were subjected to silica gel column chromatography eluting with methanol:ethanol:n-hexane (1:2:10) to yield more than 5 fractions based on TLC.

African catfish (Clarias gariepinus)

African catfish (*C. gariepinus*) is one of the common freshwater fish species was used in this study. African catfish (average weight (50±2) g; length (16.2±2.8) cm) were obtained from a private fish farm in Pare, Kediri, Indonesia. They were carefully transferred to 10 aquariums (50 L). The fish was acclimatized under aerated conditions for a period 7 days at (28.0±2)^{o}C. Fish were fed with a commercial catfish feed at *ad libitum* twice daily during the acclimatization period.

Experimental design and fractions injection

As many as 90 African catfish were randomly divided into 10 groups. Each group of 9 fishes was divided into three equal triplicate subgroups. Each aquarium was supplied with aeration by aquarium aerators and filtration. The fishes were fed *ad libitum* twice daily at 9.00 and 17.00. Water exchange (50%) was done every three days and water quality was monitored throughout the experimental days at three days intervals. The

temperature was (28.0±2)^{o}C, pH 6.5±1.5, dissolved oxygen concentration (5.3±0.2) mg/L.9 fishes was not injected as control and 9 fishes from each group were intramuscularly injected with 9 different fractions respectively at dose 0.1 ml fraction/ 50 g fish based on[7].

Collection of blood and determination of leucocyte

In Day 3 and Day 7 of experimental days, 3 fishes from each group were randomly selected for blood collection. 3.8% trisodium citrate was used as an anticoagulant. Blood was collected from the caudal vein with a 1 ml plastic syringe with trisodium citrate washed before. The blood was then transferred immediately to a microtube and shaken gently. Then, it was used for determination leucocyte using a haemocytometer following the method of[6].

Characterization of C.papaya leaves fraction

UV-visible spectrophotometer analysis

An ELICO SL-159 UV–visible spectrophotometer (Andhra Pradesh, INDIA) was employed for the spectrometric analysis of *C. papaya* L. leaves fraction 9. The reduction of *C. papaya* L. leaves fraction was measured periodically at 200–800 nm. A spectrum of *C. papaya* L. leaves fraction was plotted with wavelength on x-axis and absorbance on the y-axis. The absorbance peaks can be observed.

Fourier transform infrared (FTIR) analysis

5 ml of *C. papaya* L. leaves fraction 6 was analysed by Fourier transform infrared spectrum (FTIR), Nicolet Avatar 660 (Nicolet, USA).

Gas Chromatography –Mass Spectrophotometry (GC-MS) analysis

Secondary metabolites quantification and identification of *C. papaya* L. leaves fraction were analysed using GC–MS. GC-MS analyses were performed using a VG Quattro Mass spectrometer (VG Micromass, UK), equipped with a Supelco SPB-5 capillary column (length: 30 m; ID: 250 mm; film thickness: 1.4 mm). The helium carrier gas was set to a column head pressure of P ¼ 100 kPa. The inlet temperature was set at 280^{o}C while the oven temperature was initially at 100^{o}C (held for 3 min) and then increased to 315^{o}C at 20^{o}C/min. The mass spectrometer was operated in the positive electron impact mode with an ionization energy of 70 eV. The ion source temperature was 190 ^{o}C at a pressure of 10E-4 Torr. Detection was performed in selective ion monitoring (SIM) mode and peaks were identified and quantitated using target ions.

Statistical Analysis

Data for leucocytes number were analysed using one-way analysis of variance (ANOVA) of SPSS version 20. Significance was tested at 5% level

Results

Phytochemical identification

Phytochemical identification of *C. papaya* ethyl acetate leaves extracts to result in active compounds such as tannins, alkaloids, flavonoids and triterpenoids (Figure 1).

Tannins + Alkaloids + Flavonoids + Triterpenoids +++

Figure 1. Phytochemical identifications of C. papaya L. ethyl acetate leaves extracts

Leucocytes Parameter

The leucocytes of African catfish were injected with 9 different fractions of *C. papaya* L. leaves are shown in Table 1. Significantly highest leucocyte count was recorded infraction 9 of *C. papaya* L. leaves whereas fraction 9 and fraction 5 have significant difference among control groups and the other fractions.

Table 1. African catfish leucocytes (x10^3 cells/ml) were injected with 9 different fractions of C. papaya L. leaves and control

	Days	
Fractions	**3**	**7**
Control	29,67±2,08	28,33±2,52
1	38,00±2,00	38,00±1,73
2	57,33±2,52	54,67±2,52
3	48,67±2,52	39,33±1,53
4	45,67±1,53	46,33±2,52
5	69,67±2,08*	70,33±2,52*
6	27,00±2,00	27,67±2,52
7	45,67±2,52	49,33±1,53
8	58,00±2,05	56,00±1,63
9	78,00±2,00*	80,00±1,73*

*: (P<0,05).

Figure 2. Nine fractions of C. papaya L. ethyl acetate extract from silica gel chromatography column with mobile phase mixture methanol:ethyl acetate:N-hexane (1:2:10)

Characterization of C. papaya L Leaves Fraction 9

UV-visible spectrophotometer

UV-vis analysis of fraction 9 results in many absorbance peaks. Based on the standard was dissolved in eluent mixture, fraction 9 contains quercetin (flavonoid), triterpenoid and alkaloid. Quercetin was absorbed in wavelength 249, 240, 221, 219 and 204 nm. Triterpenoid was absorbed in wavelength 436, 261, 223 nm; besides alkaloid absorbed at wavelength 213 nm. Shown in Fig 3.

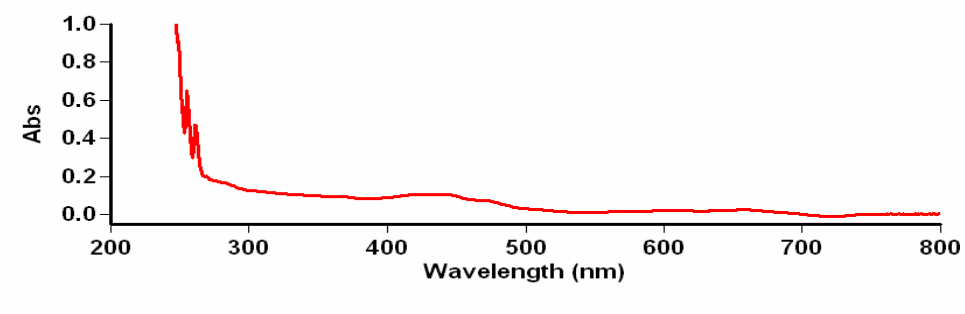

Figure 3. UV- Vis analysis of C. papaya L. leaves fraction 9

Fourier Transform Infrared (FTIR)

FTIR spectrum was analysed for identification of different biomolecule adsorbed on the fraction 9. The FTIR spectrum of synthesized fraction 9 peaks was observed at 3463,958 cm-1 which are associated with N-H stretching, O-H groups, H-bonded alcohols, phenols, and carboxylate acid. Peaks were observed at 2927,351 cm-1 which are associated with an aldehyde, alkane C-H stretching and ketones, at 1737,584 cm-1 which associated with alkane C-H stretching, at 1642,675 which associated with alkene C=C stretching, C==N stretching, N-H primary amine, and amide stretch. Meanwhile, peak was observed at 1574,113 cm-1 with alkane C-H stretching, alkene C=C stretching, C==N stretching, primary and secondary amine C-N stretching and amide. 1384,386 cm-1 which are associated with O-H groups, H-bonded alcohols, phenols and carboxylate acid Shown in Figure 4.

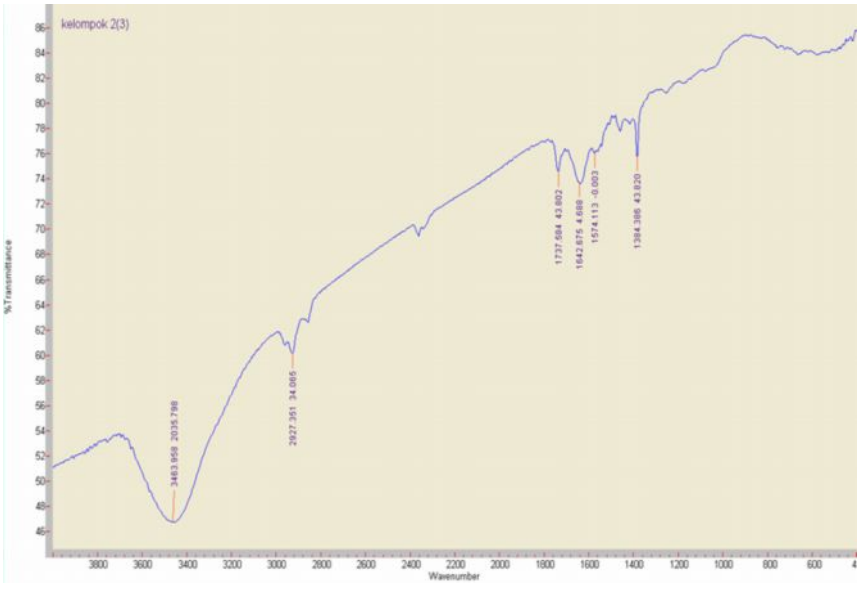

Figure 4. FTIR analysis of C. papaya L. leaves fraction 9

GC-MS Analysis

GC-MS analysis detected 11 active compounds in *C. papaya* L. leaves fraction 9. The identification of each peak was achieved by comparing the retention time (rt) and mass spectra of the compounds in the fraction 9 to the database library. Peak 10 was assumed as the major active compound due to has the highest peak with retention time 23.417 It was formed by molecules of hexanedioic acid, bis(2-ethylhexil) ester and hexanedioic acid, dioctyl ester. Shown in Figure 5.

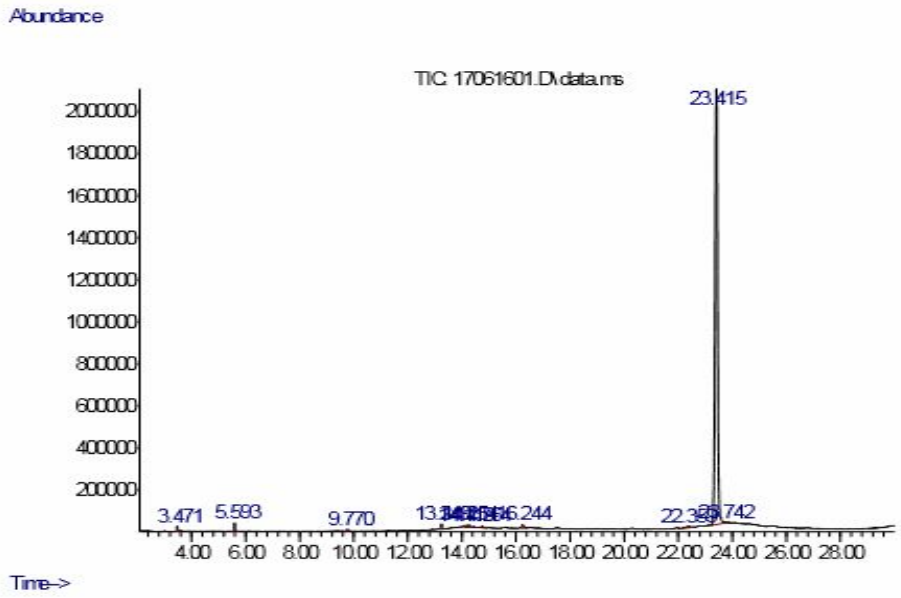

Figure 5. GC-MS analysis of C. papaya L. leaves fraction 9

Discussion

Phytochemical identifications have indicated that *C. papaya* L. leaf extract contains chemical compounds such as alkaloids, tannins, flavonoids, and triterpenoid. These compounds can affect various biological processes in the body in ways that might have harmful or beneficial effects (Baskaran *et al*., 2012; Aruljothi).

In this study, *C. papaya* L. leaves ethyl acetate extracts was subjected to silica gel column chromatography with mobile phase mixture of methanol:ethanol:n-hexane (1:2:10) yielded 9 fractions. On day 3 and day 7 observation, among control and 8 other fractions, fraction 9 was increasing African catfish leucocytes number 78,00±2,00 and 80,00±1,73(x 10^3 cells/ ml) respectively. These were still normal values 60-150 (x 10^3 cells/ ml) of African catfish leucocytes. The UV- Vis analysis of fraction 9 shown that fraction 9 contains active compound such as flavonoid especially quercetin (at wavelength 249, 240, 221, 219 and 204 nm) based on the standard. Besides, it contains triterpenoid in wavelength 436, 261, 223 nm and alkaloid at wavelength 213 nm (Harborne , 2006). This analysis similarly with FTIR analysis which the major active compound of fraction 9 is triterpenoid. Triterpenoid is active compound was majority formed by alcohol, aldehyde and carboxylate acid (Harborne, 2006).

GC-MS analysis resulted in the major active compound of *C. papaya* L. leaves fraction 9 was hexanedioic acid, bis(2-ethylhexil) ester and hexanedioic acid, dioctyl ester. These active compounds are triterpenoid derivatives (Harborne, 2006).

These results indicate that *C. papaya* L. leaves fraction 9 contains active compounds hexanedioic acid, bis(2-ethylhexil) ester and hexanedioic acid, dioctyl ester that are triterpenoid derivatives. These indicate that there is an increase on the leucocytes number of African catfish. Besides, it has beneficial effects on fish health and enhance the immune system (leucocytes number) and hence they could play an important role in preventing disease outbreaks in aquaculture systems. However, in most cases, mechanisms responsible for the physiological answer in fish are still unknown.

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