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# BIOACTIVITY GUIDED FRACTIONATION OF *Spondias mombin* (ANACARDIACEAE) LEAF EXTRACTS AGAINST *Artmia salina*

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## ABSTRACT

Plants extracts are known for their various ethno-medicinal applications, especially in the cure and management of inflammation and cancer. In this study, the crude methanolic extract of leaves of the plant *Spondias mombin* exhibited activity against *Artemia salina* in a preliminary assay for anti-inflammatory and anticancer activity. It was further observed that fractions of the extract obtained from vacuum liquid chromatography and adsorption column chromatography gave improved toxicity against this organism. The fraction from column chromatography with the highest activity (the CCG fraction) was further separated by adsorption column chromatography to give five fractions (P45, P46, P47, P48, and P49) and their toxicity and chemical composition were determined. Fractions P47, P48 and P49 exhibited the highest toxicity with $LD_{50}$ and $LD_{90}$ at 24 hr of 3.37±1.02, 3.33±0.62, 1.83±0.30 $LD_{90}$µg/ml and 25.99±2, 55, 28.29±4.43 13.59±4.37 µg/ml respectively**.** Fractions P47 and P48 were very similar in their activities and were slightly less active than the G sub-fraction but the P49 was most active of them all with the $LD_{50}$ and $LD_{90}$ of 1.83±0.30 and 13.59±4.37 µg/ml respectively. The GC-MS of P49 than P47 and P48 revealed the presence of the following bio active substances: (z-(13, 14 -epoxy) tetradec-11-en-1-ol acetate, (2-methyl-7-oxo-1.7-dihydro pyrazolo(1,5-a) pyrimidine-5-carboxylate, dibutyl phthalate, 1, 2-benzenedicarboxylic acid bis (2methyl propyl) ester, benzenedicarboxylic acid bis (2ethyl hexyl) ester and allylamine, 3-chloro-N-isopropyl-2-methyl-,(Z). The fractions contain bio active substances that maybe used in the management of cancer.

**KEY WORDS:** *Spondias mombin* leaves, anti-inflammation, cancer, *Artemia salina,* pyrimidine-5-carboxylate, allylamine,

## INTRODUCTION

Cancer is one of the leading causes of mortality in the world today. The death due to cancer is projected to be 12 million per year by 2030. Tumor promoter causes chemical oxidative stress in cells. In the cell, oxidative stress generates a lot of reactive radicals such as $O^-$ and $OH^-$ that attack cellular targets such as DNA, protein as well as lipid-rich membranes. This process leads to inflammation and tumor growth if there are no antioxidant molecules or enzymes to counter these free radicals (Jifa *et al*., 1999).

Nature has provided the source of treatments for different ailments including cancer in plants and many other natural products. Recent studies have shown that the use of various crude extracts or isolated purified compounds from plants can effectively control cancer and some inflammatory diseases (George *et al*., 2015). *Spondias mombin* Linn (Family Anacardiaceae), is a fructiferous tree that thrives in the rainforest and coastal areas of Africa It is widely found in tropical America, Asia and Africa, and has been recently cultivated in commercial quantities in Mexico (Leon and Shaw, 1990). It is variously known as hog plum or yellow mombin in English, Ijikara Ichikara or Uchakuru in Igbo, Akika etikan, Iyeye in Yoruba, Tsardarmasar in Hausa and Igongo or Ichankla in Idoma. It is a medium sized but occasionally large tree commonly found in the forest and savanna regions of Nigeria (Chukwuka and Thomas, 2008). The aromatic fruits, hung in numerous branched clusters, are, ovoid and oblong in shape (Corthout *et al*., 1994).

Nigerian plants, particularly *S. mombin,* have been studied in the search for cytotoxic plants with potentials for the management of inflammation and cancer. This plant is used traditionally for the management of inflammation and cancer (Ibikunle *et al*., 2017). Also, in Nigeria it has several medicinal applications: as an abortifacient and anti-inflammatory, a childbirth aid, a diuretic, expectorant, and febrifuge, and a hemostat, laxative, and oxytocic (induces labor). It is also employed for the treatment of burns, cholera, cough, cuts, diarrhea, dizziness, eye ailments, fever, gonorrhea, malignant tumors, nervous disorders, sore throat, sores, stomach pains, tapeworm, wounds and yaws (Taylor, 2004, Senthil *et al*., 2013, 1992, Chukwuka and Thomas, 2008).

Previous workers found that the crude methanol extract, and a fraction derived from it by partition exhibited cytotoxicity against *Artemia salina*. Vacuum liquid chromatography of this fraction gave three fractions of higher activity (Ibikunle *et al*., 2017). The crude extract SM0 and n-hexane fraction SM1 had cytotoxicity against *A. salina*. The SM1 when subjected to vacuum liquid chromatography and fractionated into n-hexane (S1-1), chloroform (SM1-23) and ehtylacetate (S1-4) were active (Ibikunle *et al.*, 2017). Due to the improved activities, effort was made to purify further, some of these fractions. This research was undertaken in order to further resolve of one of these active fractions (SMI-23) by adsorption column chromatography, and determine the activity of the fractions obtained against *A. salina,* followed by the separation and identification of their chemical constituents by gas liquid chromatography-mass spectrometry. The possibilities for the utilisation of these compounds in various areas, including as antioxidant and anticancer agents are discussed.

## MATERIALS AND METHODS

### *Materials*

The leaves of *Spondia mombin*, were collected from Odo-Owa, Oke Ero Local Governmemt Area of Kwara State, Nigeria. The plant with voucher specimen number IFE17284 was authenticated by Mr. B. E, Omomoh of the Department of Botany, Obafemi Awolowo

University, Ile-Ife, Osun State, Nigeria.

The materials for the toxicity bioassay included *Artemia salina* cyst (egg), sea water, aerator, capillary tubes and 60 watt bulbs. The *A. salina* cyst (egg) and sea water were obtained at the Nigeria Institute of Oceanography and Marine Research, Lagos.

Silica gel Kieselgel for column chromatography (60, mesh 0.015-0.04mm from Machery Nagel Germany, Aluminium sheets precoated with silica gel 60 $F_{254}$ plates of 1.00 mm thickness, for preparative thin layer chromatography (Machery Nagel Germany). UV lamp model UVGL-15 at 254nm and 365nm. Gas Chromatograph-Mass Spectrometer (Agilent Technologies Model 7890 Series GC System equipped with an Agilent Technologies 5975 MS detector (EI mode, 70 eV). All reagents were analytical grade.

***Methods***

The extract was evaporated to dryness *in-vacuo* using a rotary evaporator (state the model and manufacturer) and coded $SM_0$. The crude extract ($SM_0$) was successively partitioned into n-hexane, chloroform, ethyl acetate and water then coded as $SM_1$, $SM_2$, $SM_3$ and $SM_4$; respectively in increasing order of polarity.

***Vacuum Liquid Chromatography***

The n-hexane extract (SM1, wt 33.00 g) was subjected to vacuum liquid chromatography, with gradient elution, using the solvent system n-hexane, chloroform, ethyl acetate and aqueous. This gave four fractions with yields as follows: SM1-1 (6.75), SM1-2 (4.04 g), SM1-3 (5.42 g) and SM1-4 (14.98 g). Bulked SM1-2 and SM1-3 of weight 9.46 g, was coded SM1-23.

***Column Chromatography of SMI-23***

The SM1-23 fraction was separated by column adsorption chromatography on 60 –120 mesh silica gel (Machery Nagel Germany) as the stationary phase and n-hexane and chloroform/ ethyl acetate (1:1 v/v) as the mobile phase. Qualitative TLC analysis of the eluted fractions was carried out on 1.00 mm thick silica gel 60 $F_{254.}$ Resolved constituents were viewed under UV light at 254 nm and 365 nm. Based on the TLC results fractions from column chromatography were combined base on their $R_f$ values. The bulked fractions obtained were coded CCA-CCG.

***Column Chromatography of CCG***

The chloroform/ ethyl acetate fraction (CCG) was separated by column adsorption chromatography on silica gel (Kieselgel 60, mesh 0.015-0.04mm, Machery Nagel Germany). With an n-hexane/chloroform gradient as the mobile phase, the dried fraction (CCG) was adsorbed on 2 g of silica gel and air dried. The dried sample was loaded on a column and eluted with different solvent ratios consisting of n-hexane, chloroform, ethyl acetate and aqueous. Qualitative TLC analysis of the eluted fractions was carried out on 1.00 mm thick silica gel 60 $F_{254.}$ Resolved constituents were viewed under UV light at 254 nm and 365 nm. Based on TLC results, different fractions from column chromatography were bulked and coded P45, P46, P47, P48 and P49.

***Artemia salina bioassay***

The crude extracts and fractions were screened for cytotoxicity using the procedure of McLaughlin *et al.* (1998) as follows:

***Preparation of shrimp nauplii***

*Artemia salina* cyst (2.0 g) were placed in 800 mL sea water (salt solution: 38.0 g NaCl/litre of distilled

water) in a 1L conical flask placed under 60 watt bulbs with an aerator and aeration stone fitted with a rubber capillary. The aeration and the illumination were done for 48h for the hatching of the *A. salina* cyst into shrimp nauplii. The shrimps were left under aeration and illumination for another 48 hours before the cytotoxicity bioassay (Mclaughlin *et al*., 1998).

***Preparation of samples for brine shrimp cytotoxicity bioassay***

The sample (20 mg) was dissolved in 20 mL of solvent to give 1000 µg/ mL. Serial dilutions of the extract were prepared by taking 0.2 ml from 1000 µg/mL into 1.8 mL solvent to give 100 µg/mL Furthermore, 0.2 mL was taken from 100 µg/mL into 1.8 mL solvent to give 10µg/mL (recommended final dilutions of 10, 100, 1000 µg/mL). Each of the samples was prepared by weighing 2.0 mg into 1.8 mL of the solvent to give 100 µg/mL and dilutions of 10 and 1.0 ppm were prepared from it.

***Cytotoxicity bioassay using shrimp nauplii***

Sea water (3 mL) was dispensed into a calibrated 5ml vial and then 10 shrimp nauplii were counted into it. A transparent glass capillary was used for the counting into the vials. To each of the vials the 0.5 mL of the prepared extract or fraction or isolate was added, and then made up to 5 ml with sea water. All the bioassay experiments were performed in triplicate. After 24 hours the number of shrimps nauplii that survived (lethality estimate) and number of shrimps nauplii with sluggish motion (sedative estimate) were determined. The analyses of the lethality data and the $LD_{50}$ and $LD_{90}$ at 95% confidence limit were carried out using the Finney Computer program (Ibikunle *et al.,* 2017).

***GC-MS Separation and Identification***

The GC-MS analysis was carried out using Agilent Technologies 6890N Network GC system coupled with mass spectrometer detector 5975. The column was 15 m in length, 0.25 mm in internal diameter and 0.25 µm in thickness. Helium gas was used as carrier gas at constant flow rate of 1ml/ minute. Injector temperature was set at 100 ºC and maximum temperature of 335ºC. The separation was done using Agilent capillary GC (BD-624) column in isocratic run with ramps rate of 10ºC /min and with a detector temperature of 250ºC. Each dried sample was dissolved in 5 ml of chloroform and 2 µL of this solution was employed for the GC – MS analysis. The sample (1µl) was injected using syringe size 10.0 µL through an injector plunger speed fast.

## RESULTS AND DISCUSSION

The results of biotoxicity of column chromatograph from the SM1-2 and SM1-3 were similar and were bulked and coded SM1-23. The SM1-23 was further purified by column chromatography which gave the fractions CCA-CCM. CCG had very good yield (4.3756 g) and good activity of $LD_{50}$ of 2.60±0.42, µg/ml. Due to these observations, it was subjected to further column chromatography to give fractions P45, P46, P47, P48 and P49.

Table 1: Biotoxicity of fractions CCA-CCM against *Artemia salina*

| Code | LD50 µg/mL | LD90 µg/mL | Wt. (g) |
|---|---|---|---|
| SM1-23 | 6.03±0.37 | 46.37±1.78 | |
| CCA | 103.03±29.40 | 658.101±207.20 | 0.321 |
| CCB | 157.85±5.19 | 814.13±298.37 | 1.3354 |
| CCC | 206.36±90.35 | 1040.20±411.38 | 0.0653 |
| CCD | 44.09±13.79 | 207.31±84.17 | 0.0855 |
| CCG | 2.60±0.42 | 19.98±5.13 | 4.3756 |

Biotoxicity values for CCG, P45, P46, P47, P48 and P49 against *A. salina* are given in Table 1, and indicate that the activity of the CCG was not based on synergy**.** There were specific compounds responsible for the activities**.** The P45 did not exhibit any activity while the activity of P47 and P48 were similar with the $LD_{50}$ and $LD_{90}$ at 24 hr 3.37and 3.33 µg/mL and 25.99 and 28.29 µg/mL respectively. P49 had an $LD_{50}$ of 1.83 µg/mL which revealed a higher toxicity than CCG with a value of 2.60 µg/mL**.** The P47 and P48 were similar in activity and were slightly less active than CCG. P49 was the most active of them all, with $LD_{50}$ and $LD_{90}$ of 1.83 and 13.59 µg/mL respectively (Table 2).

Table 2: Toxicities of SMI-23, CCG, P45, P46, P47, P48, and P49 against *A. salina*

| Fractions | $LD_{50}$ (µg/mL) | $LD_{90}$ (µg/mL) |
|---|---|---|
| SM1-23 | 6.03±0.37 | 46.37±1.78 |
| CCG | 2.60±0.42 | 19.98±5.13 |
| P45 | -ve | -ve |
| P46 | 121.58±5.11 | 769.06±56.02 |
| P47 | 3.37±1.02 | 25.99±2,55 |
| P48 | 3.33±0.62 | 28.29±4.43 |
| P49 | 1.83±0.30 | 13.59±4.37 |

The GC-MS of P46, P47, P48 and P49 spectra were recorded in figure1, 2, 3 and 4. The chemical constituents revealed by each were extracted and recorded in Table 3.

The constituents of the he active fractions (P46, P47, P48 and P49) were successfully identified GC-MS analysis. (Figures 1-4 and Table 3).

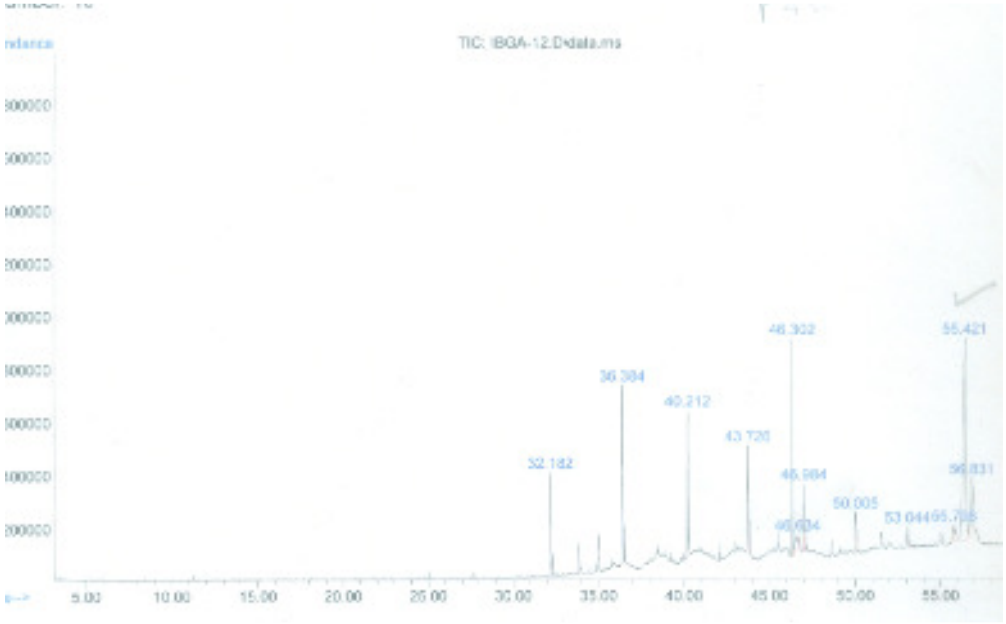



Fig. 1: Gas chromatogram of P46

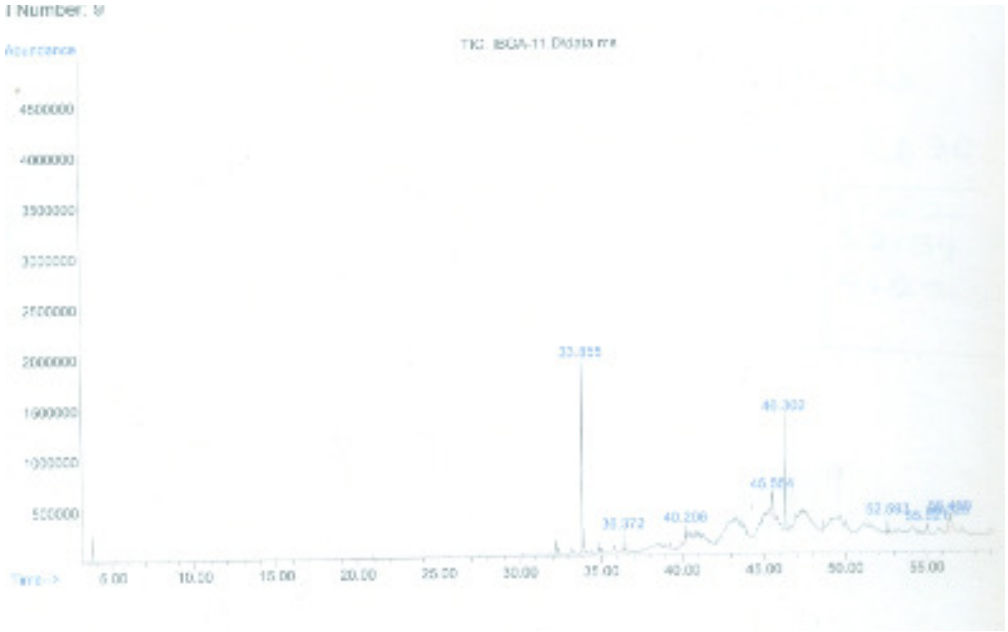



Fig. 2: Gas chromatogram of P47

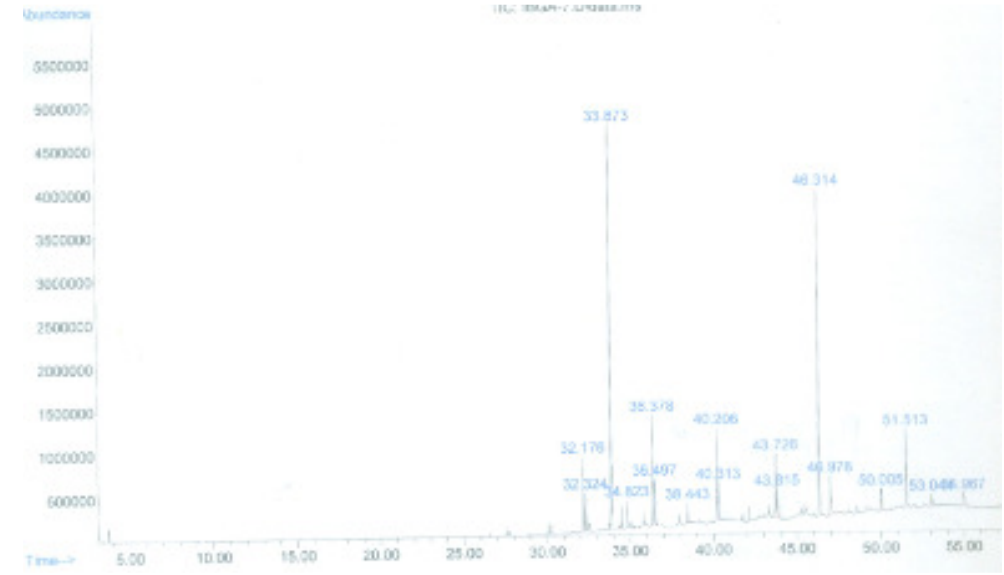



Fig. 3: Gas chromatogram of P48

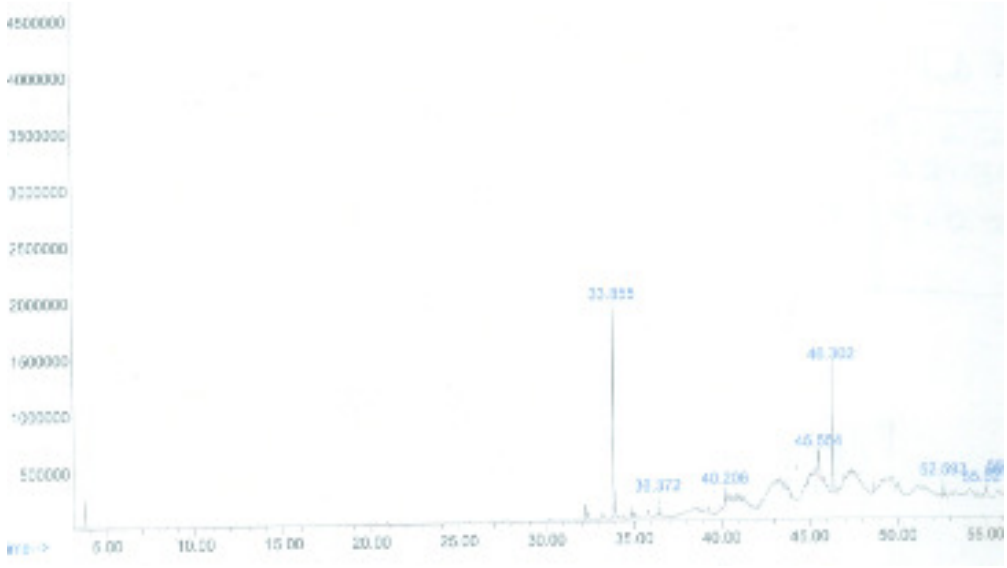
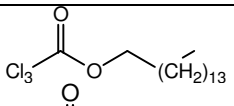
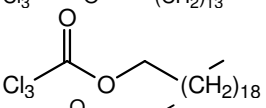
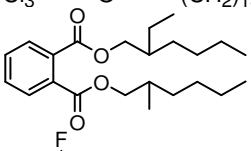
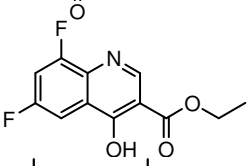
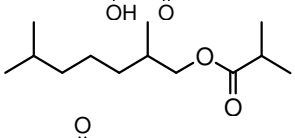
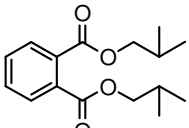
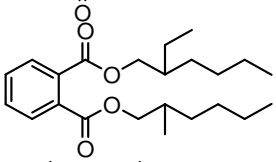
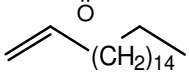
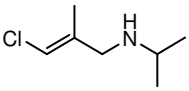
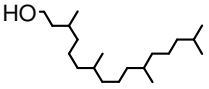
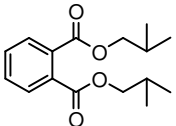
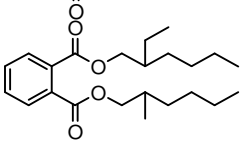
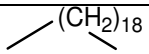
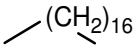
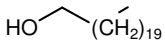
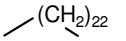
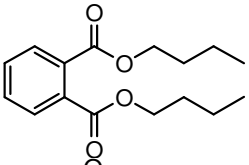
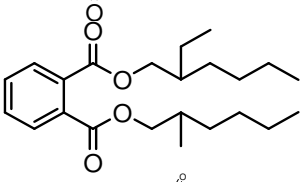
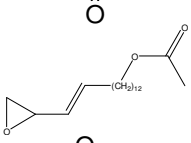
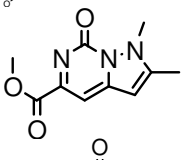
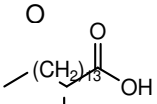
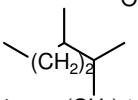
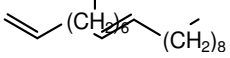



Fig. 4: Gas chromatogram of P49

Table 3: Structures, retention times, composition, activities, and possible applications of the constituents of the active fractions

| Fraction | Name of the Compound | Structure of the compound | Retention Time (min) | % | Activity/uses |
|---|---|---|---|---|---|
| P46 | Trichloroacetic acid, pentyadecyl ester |  | 36.384 | 8.56 | Antiaging and sun protection agent, cytotoxic to Hela and MCF-7 (Hsouna *et al*., 2019). |
| | tetradecyl Trichloroacetic acid ester |  | 43.726 | 5.47 | |
| | bis (2- ethylhexyl) phthalate |  | 46.302 | 11.16 | Plasticizer compound |
| | 3-quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-ethyl ester |  | 55.798 | 2.17 | No activity reported |
| | citronellyl isobutrate |  | 56.421 | 40.31 | Anti-inflammatory and food flavor (Jose *et al.,2013*) |
| P47 | 1, 2-Benzenedicarboxylic acid bis (2methyl propyl) ester |  | 33.879 | 43.74 | They are anti-inflammatory in rat (Costanito et al 1993) |
| | Benzenedicarboxylic acid bis (2ethyl hexyl) ester |  | 46.326 | 33.20 | Plasticizer compound |
| | 1-octadecene |  | 36.384 | 7.12 | Hepatoprotective, anti androgenic, nematocide 5-alpha reductase inhibitor; antihistamin, anticancer. |
| | Allylamine, 3-chloro-N-isopropyl-2-methyl-(Z)- |  | 38.954 | 5.41 | For pancreatic cancer, ovarian and stomach cancer (Sheng-Teng et al., 2006). |
| | Phytol |  | 56’368 | 10.53 | Antimicrobial, anticancer, antioxidant, duretic (Sivagurunathan and Xavier, 2014)**,** |
| P48 | 1,2-Benzenedicarbxyic acid bis (2methyl propyl) ester |  | 33.879 | 28.74 | anti- inflammatory in rat (Costanito et al 1993). Plasticizer compound |
| | bis(2- ethylhexyl) phthalate |  | 46.302 | 22.16 | Anti-microbal and ant-oxidant. Inhibits |

| | | | | | |
|---|---|---|---|---|---|
| | Nonadecane, |  | 32.176 | 4.53 | Hela and MCF-7 cell lines (Hsouna *et al*., 2019; Sylvester et al.,2006) |
| | Octadecane |  | 32.324 | 2.31 | |
| | Heneicosanol , |  | 40,206 | 5.55 | |
| | Tetracosane |  | 51.513 | 6.27 | |
| P49 | Dibutyl phthalate |  | 33.855 | 36.44 | Antibacterial, antifouling, anti-inflammatory in rat (Costantino et al., 1993, Senthil et al., 2016) |
| | bis(2- ethylhexyl) phthalate |  | 46.302 | 23.98 | Plasticizer compound |
| | z-(13, 14-epoxy) tetradec-11-eno-ol acetate |  | 52.593 | 4.90 | Antioxidant |
| | Methyl, 2methyl-7-oxo-1,7-dihydropyrazolo(1,5-a) pyrimidjne-5 carboxylate |  | 55.021 | 4.55 | Alkaloid |
| | hexadecanoic acid, tetradeceyl ester |  | 56.465 | 9.35 | Antimicrobial, antioxidant, and anticancer (Sylvester *et al*., 2006) |
| | Hexane, 2, 3-dimethyl |  | 45.554 | 5.80 | |
| | 8-octadecenal |  | 56.36 | 5.34 | |

The P46 was different from P47, P48 and P49 in chemical constituents and biotoxicity. P46 has the citronellyl isobutrate as its dominant constituent with 40.31% (Fig.5, Table 3), with lower content of trichloroacetic acid pentyadecyl ester and tetradecyl trichloroacetic acid ester. Two major compounds found in P46 that might be responsible its activity are trichloroacetic acid, pentyadecyl ester and citronellyl isobutrate the former exhibits antiaging and sun protection, properties cytotoxicity to HelaMCF-7, cells and the latter is an anti-inflammatory that also, finds application as a food flavouring (Hsouna *et al*., 2011 Jose *et al.,*2013).

The P47 and P48 (Figs 2 and 3, Table 3) contained two major compounds 1, 2-benzenedicarbxyic acid bis (2-methyl propyl) ester with retention time of 33.879 min (43.74%, 28.74%) and benzenedicarboxylic acid bis (2ethyl hexyl) ester retention time of 46.326min (33.20% 22.16%) respectively. They had $LD_{50}$ and $LD_{90}$ of 3.37±1.02 µg/ml and 25.99±2.55 µg/ml. These compounds in combination, have been reported to exhibit anti-inflammatory activity in rats (Costanito *et al.,* 1993).

Furthermore 1, 2-Benzenedicarboxylic acid bis (2-methyl propyl) and benzenedicarboxylic acid bis (2-ethyl

hexyl) have antibacterial effect (Sivakumar, 2014). The allylamine, 3-chloro-N-isopropyl-2-methyl-, (Z) - with 5.41% may be responsible for the observed level of toxicity in the fraction. Fraction P47 and P48 shows slight differences in their toxicity and this may be due to the variation in their chemical constituents reported to have antioxidant and inhibitory activity against Hela and MCF-7 cell line (Hsouna *et al.,* 2019; Sylvester *et al*., 2006).

It was observed that P49 (Figure 4, Table 3) contained phthalates similar in quantity to those found in P47 and P48. P49 contained n-dibutyl phthalate, while its isomer 2-methyl propyl) ester phthalate was present in P47 and P48. The dibutyl phthalate had a concentration of 36.44% (Table 3). P49 exhibited a higher toxicity than P47 or P48; this might be due to the presence of the acetate esters z-(13, 14 -epoxy) tetradec-11-en-1-ol acetate and 2-methyl-7-oxo-1.7-dihydro pyrazolo (1, 5-a) pyrimidine-5-carboxylate (a derivative of DNA). These compounds have been reported to exert cidal effect on *A. salina* (Sheng-Teng *et al*., 2006). In this study the possible compounds that could cause the toxicity are 2H-1-benzopyran-6-ol,3,4-dihydro 2,8 dimethyl-2-(4, 8, 12, trimethyl) tridecyl (tocopherol), an antioxidant (Shwetha *et al.*, 2011), z-(13, 14-epoxy) tetradec-11-eno-ol acetate, and 2-methyl-7-oxo-1,7-dihydropyrazolo(1,5-a) pyrimidine-5-carboxylate, hexadecanoic acid, tetradeceyl ester.

## CONCLUSION

The bioactivity of fractions derived from the crude methanolic extract of *Spondias mombin* against *Artemia salina* Increased with increasing purification. The major compounds in the active fractions from adsorption column chromatography were 2-methyl-7-oxo-1.7-dihydro pyrazolo (1,5-a) pyrimidine-5carboxylate, dibutyl phthalate, 1, 2-Benzenedicarbxyic acid bis (2methyl propyl) ester, benzenedicarboxylic acid bis (2-ethyl hexyl) ester and allylamine, 3-chloro-N-isopropyl-2-methyl-(Z). Their high content of 1, 2-Benzenedicarbxyic acid bis (2methyl propyl) ester, dibutyl phthalate and 1,2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester in the more active fractions, P47, P48 and P49, coupled with evidence from the literature, suggest that these compounds played a major role in the activity against *A. salina* and could form the basis for the anti- cancer role of the leaves of *S. mombin* in ethno -medicine.

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