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In vivo hepatoprotective and *in vitro* antimicrobial potential of *Ceasalpinia bonduc* (Linn): Pharmacological correlation with identified phytochemicals

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Abstract: The *in vivo* hepatoprotective potential of methanolic extract of *Ceasalpinia bonduc* (CBLM) has been explored against carbon tetrachloride (CCl_4) induced acute liver injury in rats. Treatment of plant extract on CCl_4 intoxicated liver significantly reduced the hepatoxicity, along with serum enzymes GPT and GOT. To explore the chemical constituents from CBLM extract, it was fractionated into non-polar to moderately polar fractions (CBLM-H, CBLM-HEt, CBLM-Et, CBLM-EtM, CBLM-M) and subjected to GC/GC-MS analysis. Altogether twenty seven (~71%) phytochemicals were identified from different fractions by using Electronic Mass Spectral Library GC-MS (NIST 20). Out of which twenty one are first time reported from *Ceasalpinia bonduc*, fourteen from genus Caesalpinia and ten from family Fabaceae. The identified phytochemicals 2-ethyl-2-hydroxy-1,3-dimethylcyclopentanecarboxylic acid, ethyl ester (21) and 1,3,5-triazine-2,4-diamine,6-hydroxy-N,N-dicyclohexyl (23) are first time identified as plant metabolites. To explore the antimicrobial potential four strains of Gram-positive and eight strains of Gram-negative bacteria were used along with pure cultures of five saprophytic fungus (molds) and two strains of yeast were utilized. CBLM-H and CBLM-HEt were exhibited praiseworthy antimicrobial potential. CBLM-H showed complete growth inhibition of *P. mirabilis and V. cholerae* at the concentration of 0.1µg/mL while CBLM-HEt at 0.05µg/mL halted the growth of *S. aureus.*

Keywords: Serum GPT and GOT activities, immunomodulatory activity, toxicity, antimicrobial activity, GC/GCMS analysis.

INTRODUCTION

Hepatotoxicity is one of the most lethal diseases with a staggering figure of 550 million people in world. In developing countries more people are suffering from hepatitis, while in Pakistan nearly 35 million people are supposed to be infected with hepatitis (Ali *et al*., 2014). Long term use of drugs and contact with environmental toxins e.g., carbon tetrachloride, paracetamol, alcohol, thioacetamide, etc. causes hepatitis along with its different types of complications like cirrhosis or liver cancer (Sreelatha *et al*., 2009). These toxicants causes the production of reactive oxygen species (ROS) which have that ability to bind with polyunsaturated fatty acids along with the formation of different types of radicals for lipid peroxide, resulting in cell membrane damage and variations in activity of enzymes (Weber *et al*., 2003), and subsequently induce hepatic injury, inflammation, necrosis (cell death caused by loss of membrane) and apoptosis (programmed cell death) (Lin *et al*., 2009). Free radicals scavenging by antioxidants can decrease liver tissues fibrosis but it is also a responsible factor in a progressive decline in the function of immune system (Sreelatha *et al*., 2009). The present study explored the hepatoprotective potential of the methanolic extract of leave of *Ceasalpinia bonduc* against carbon tetrachloride (CCl_4) induced hepatoxicity in rats because CCl_4-induced liver injury are quite similar to acute viral hepatitis (Ali *et al*., 2014). *C. bonduc* (Linn) (Syn. *C. bonducella*) belongs to the family Fabaceae and 15 species of this genus caesalpinia have been heavily researched due to their folklore importance and bioactivities in which *C. bonduc* is one of them (Jing *et al*., 2019). *C. bonduc* are used traditionally for the treatment of diabetes, asthma, intermittent fever, skin diseases, convulsion, placenta removal, and mellitus along with it contains various biological activities such as antimalarial (Sundaryono *et al*., 2021), anti-inflammatory (Liu *et al*., 2021) antitumor, antioxidant, anthelmintic, insecticidal, antibacterial, antidiarrheal and cytotoxic activities (Agbo *et al*., 2015). Previously, hepatoprotective potential of hydro alcoholic and petroleum ether leaves extract against carbon tetrachloride induced hepatotoxicity were reported and hydro alcoholic extract possesses a marked hepatoprotective action (Raghuveer *et al*., 2013, Sindete *et al*., 2021). The hepatoprotective effect of methanolic

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extract of *C. bonducella* was also reported by means of paracetamol induced liver damage in rats (Gupta *et al.,* 2003). Due to the potential hepatoprotective property in the present study the hepatoprotective activity of methanolic extract of leaves of *C. bonduc* was performed against carbon tetrachloride (CCl_4) induced acute liver injury in rats, explored the immunomodulatory activity and cytotoxicity against embryo fibroblast (3T3) and Mard in-Darby Bovine Kidney (MDBK) cell lines. Phytochemical investigation leads to the identification of twenty seven (~71%) phytochemicals from different fractions of methanolic extract through GC/GCMS analysis and also evaluated the antimicrobial activity of these fractions.

MATERIALS AND METHODS

From commercial sources reagent grade solvents and chemicals have been purchased for this assay. Methanol, petroleum ether, ethyl acetate and acetone were purchased from Merck (Pvt.) Limited (Dramstadt, Germany). Fresh young leaves of C. *bonduc* were collected in February, 2013 from the nursery of University of Karachi, Pakistan. Professor Dr. Anjum Perveen (Taxanomist), Department of Botany, University of Karachi, Pakistan identified the species and voucher specimen (Number KUH-86548) has been deposited at the herbarium of same department. Fresh young leaves (60g) of *C. bonduc* were socked in methanol and obtained thick gummy liquid of CBLM extract (5.96g). The CBLM extract (3.66g) was further fractionated into five different fractions by using hexane (CBLM-H, 0.003g), hexane: ethyl acetate (1:1) (CBLM-HEt, 0.017g), ethyl acetate (CBLM-Et, 0.26g), ethyl acetate: methanol (1:1) (CBLM-EtM, 0.67g) and methanol (CBLM-M, 1.26g). CBLM extract were screened for hepatoprotective potential and their fractions (CBLM-H, CBLM-HEt, CBLM-Et CBLM-EtM and CBLM-M) were screened for antimicrobial (antibacterial, antifungal) activity and subjected to their GC/GC-MS analysis. GC-MS (Gas-chromatography-mass spectrometry) on Jeol (Japan) JMS 60H has been recorded by using acquisition of triple quadrupole (GCMS TQQQ). TQQQ working mode is EI with the ionizing potential value of 70eV. Column specifications are Agilent USB393752HHP-5MS (30m×250µm×0.25µm) while He is used as a carrier gas with the maintained pressure of 9.7853 psi. Jeol (Japan) JMS 60H column ZB-5® having EI mode with the ionizing potential value of 70eV, Phenomex (USA), Temperature of the column was tried to keep from 50 to 250°C in both cases by gradually increased with the rate of 5°C. Electronic Mass Spectral Library GC-MS (NIST 20) was helped out in solving the mass spectrum. JMS HX-110 has been used for EIMS. The methanolic extract (CBLM) was checked for hepatoprotective potential in carbon tetrachloride (CCl_4)-induced hepatotoxicity in Wistar rats along with its immunomodulatory activity on whole blood phagocytes, ROS production and cytotoxicity using two different cell lines i.e. embryo fibroblast cell line (3T3) and Mard in-Darby Bovine Kidney cell line (MDBK). Methanolic extract of *C. bonduc* (CBLM, 1.1 g) dissolved in 2ml distilled water by vigorous shaking before administration to rats. The extract was dissolved almost completely in water. Twenty male Wistar rats (220-250g) were divided into 4 groups:

Group I: Control rats
Group II: CCl_4-treated rats
Group III: (CCl_4+CBLM)-treated rats
Group IV: CBLM-treated rats

Group I was received olive oil only (1.5mL/kg body weight, b.w). Group II rats were treated with 20% CCl_4 (1.5mL/kg b.w) dissolved in olive oil. Group III rats were given CBLM extract (1g/kg b.w) 30 minutes before 20% CCl_4 treatment. Group IV rats were treated with CBLM extract (1g/kg b.w) only. All rats were received their respective treatments orally. After 24 hours of different treatment, all rats were anesthetized by intraperitoneal injection of pentobarbital sodium (60mg/kg b.w) and were sacrificed. Blood samples were collected via renal portal vein of rats and centrifuged at 5000 rpm for 10min for serum separation. Serum glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transanunase (GOT) and creatinine levels were measured by standard techniques using the Reflotron® Plus Dry Chemistry Analyzer (Roche Diagnostics). The extract CBLM has been tested for its immunomodulatory activity on whole blood phagocytes, ROS production and cytotoxicity using two different cell lines *i.e.* embryo fibroblast cell line (3T3) and Mard in-Darby Bovine Kidney cell line (MDBK). Adult Wistar rats (220-250g) were injected intraperitoneally with 50% CCl_4 in olive oil. *C. bonduc* was orally administered before CCl_4 treatment in Wistar rats. Twenty-four hours after CCl_4 injection, serum GPT and GOT activities, and histological changes of liver were examined. For oxidation burst ROS production, Luminol enhanced chemiluminescence assay was applied as describe by Helfand (Helfand *et al*., 1982). For acute toxicity single dose of CBLM extract (1g/Kg) orally (n =3) and i.p. (n-3) as well as multiple dose administered orally (n=2) and intraperitoneally (i.p.) (n=2) for seven days. Pure cultures of four Gram-positive bacteria i.e. *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Bacillus subtilis* and eight strains of Gram-negative bacteria i.e. *Pseudomonas aeruginosa, Enterococcus spp, Escherichia coli, Vibrio cholerae, Klebsiella spp, Proteus mirabilis, Acinetobacer spp* and *Salmonella typhi* were used as indicator strains to explore antibacterial nature of the fractions. ~~studied~~. Pure cultures of five saprophytic fungus (molds) i.e. *Aspergillus terreus, Aspergillus niger, Aspergillus flavus*, *Trichophyton spp, Penciillium spp.* and two strains of yeast fungus namely *Candida albicans* and *Candida tropicalis* were also obtained to verify the antifungal effects of test compound. Nutrient broth (Oxoid) and

Sabouraud's dextrose broth (Oxoid) were used as growth medium for bacteria and fungi, respectively. Technical agar (Oxoid) was also added as solidifying agent, when necessary in the experiment. All of the test strains were revived, sub cultured into their respective broth medium (*i.e.* Nutrient agar or Sabouraud's dextrose agar medium) and incubated for one hour at 37°C. After completion of incubation period, the turbidity of inoculated culture tubes was adjusted as per 0.5 McFarland standards (10^6 CFU/mL). The lawn of indicator strains was prepared on to the surface of respective agar medium with the help of pre-autoclaved cotton swabs under aseptic conditions. Wells (diameter = 6 mm were cut in the agar medium with the help of sterile cork-borer and filled with 100µL of the test samples, ethyl acetate and methanol (negative control) as well as chloramphenicol or nystatin (positive control). The plates were incubated for 24h at 37°C and 168h at 28°C for bacterial and fungal growth, respectively. Following that, diameter of growth inhibition haloes (area where indicator microorganism unable to grow due to susceptibility to the compounds present in sample) around the wells were observed and recorded in millimetre. The fractions exhibiting persuasive antimicrobial outcome were serially diluted in sterile nutrient broth. All the dilutions were inoculated with 1mL of the broth suspension of test bacteria and incubated overnight at 37°C. All the tubes were examined for the turbidity of the medium that indicate existence of bacteria in medium.

ETHICAL APPROVAL

The animal studies were conducted according to the ethical guidelines provided by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and approved by Institutional Animal Care and Use Committee at the International Center of Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan.

RESULTS

Serum GPT and GOT activities significantly decreased in *C. bonduc* pre-treatment rats. CCl_4-induced group showed moderate inflammation around the central vein and vacuolar degeneration. Treatment of plant extract at 1 g/kg, reduced the severity of CCl_4-induced liver intoxication. The extract-treated group showed mild inflammation around the central vein, moderate vacuolar degeneration and very minimal periportal inflammation (fig. 1). Results from the histological studies (fig. 2) were in agreement with the measured activities of serum enzymes.

Control group morphologically showed normal liver architecture in the form of hepatocytes, portal tract and central vein with no inflammation or any damage to liver tissues. However, CCl_4-induced group showed moderate inflammation around the central vein, moderate vacuolar degeneration and mild periportal inflammation. Treatment of plant extract at 1g/kg, reduced the severity of CCl_4-induced liver intoxication. The extract-treated group showed mild inflammation around the central vein, moderate vacuolar degeneration and very minimal periportal inflammation.

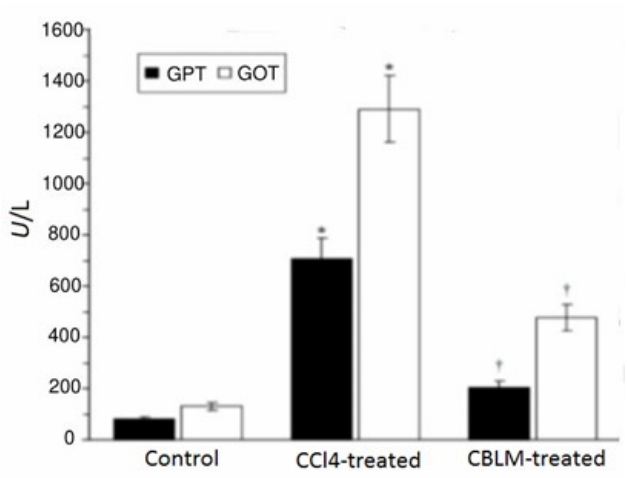

Fig. 1: Serum GPT and GOT levels in control, CCl_4-treated and CBLM-treated rats (n=5 rats per group). Values shown are mean ± SEM *p<0.001 compared with control, p<0.001 compare with CCl_4-treated rats.

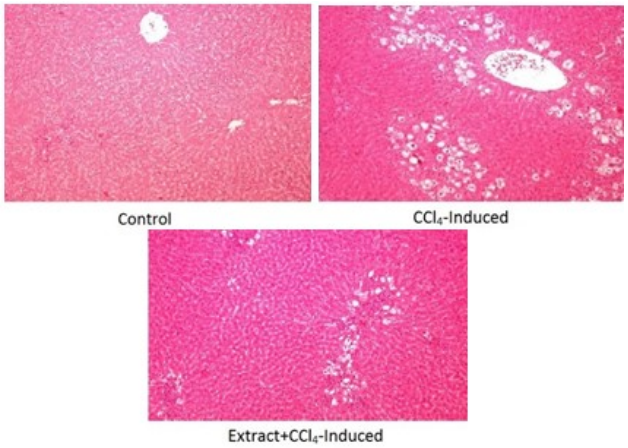

Fig. 2: Photomicrography of liver section of rats, H&E stains, magnification, 4x.

Therefore, it may be concluded that plant extract has a role on protection of inflammation and vacuolar degeneration. For the determination of acute toxicity single dose of CBLM extract (1g/kg) orally (n=3) and intraperitoneally (i.p.)(n-3) as well as multiple dose administered orally (n=2) and i.p. (n=2) for seven days but no behavioural changes or mortality occurred in rats. The tested extract did not show any inhibition on oxidative burst ROS production when tested on whole blood phagocytes after serum opsinized zymosan activation. In addition to this when studied for its effect on 3T3 and MDBK cell lines no any toxicity was

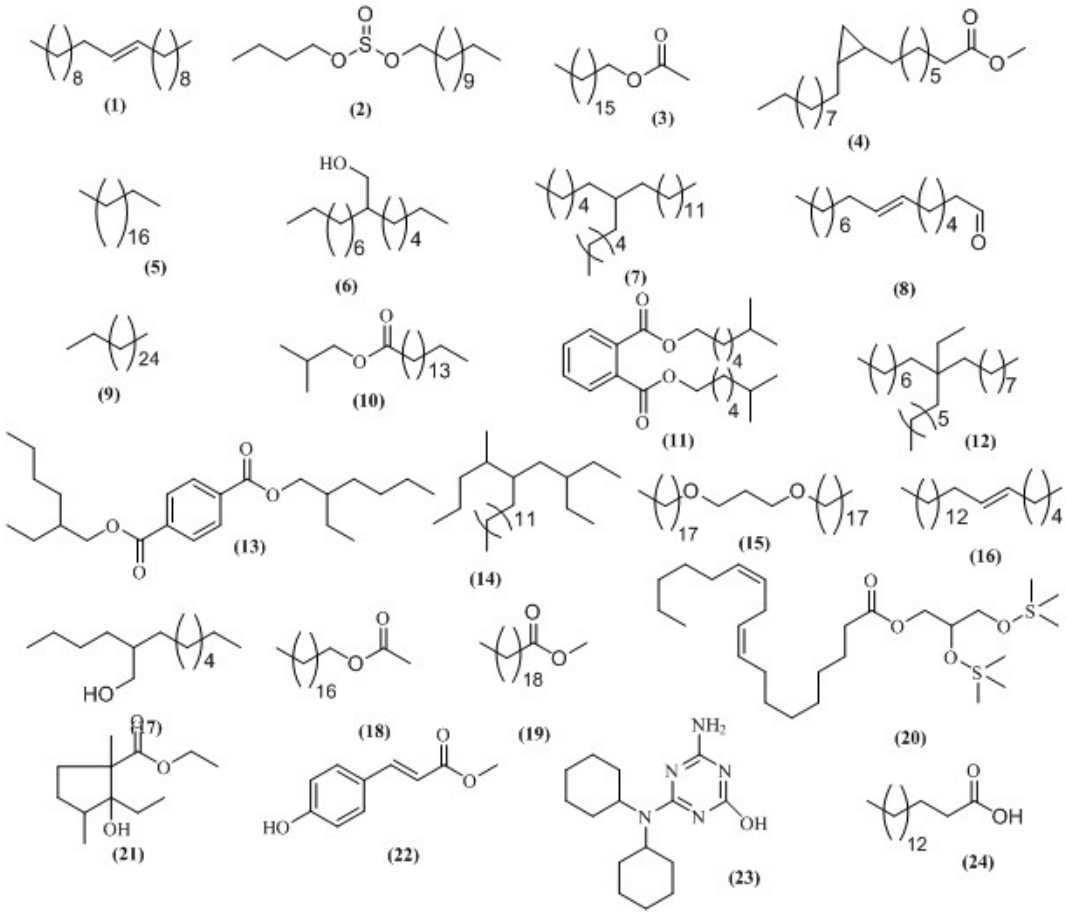

Fig. 3: Structure of the compounds identified through GC/GCMS

observed up to 200µg/mL. Methanolic extract (CBLM) and their all fractions were screened for antimicrobial (antibacterial, antifungal) potential.

For antibacterial potential four stain of Gram-positive bacteria and eight strains of Gram-negative bacteria were used (table 1). For antifungal activity five saprophytic fungus (molds) and two strains of yeast fungus were used (table 2).

CBLM-H showed potent activity as compare to the standard drug chloramphenicol against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus* with zone of inhibitions 41.60±2.05, 44.30±2.86, 31.00±0.81 and 34.60±1.24 respectively. It was also found to be potent against Gram negative *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Enterococcus Spp, Acinetobacer Spp.* and *Vibrio cholerae* with zone of inhibition 45.60±2.49, 33.66±1.88, 43.60±1.24, 50.00±0.81, 31.33±1.24, 37.60±3.39, 43.60±2.62. CBLM-HEt showed zone of inhibition 54.30±0.94, 54.00±2.16, 41.30±2.05 and 37.00±0.81 against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus* respectively. It was also showed potency against Gram negative *Salmonella typhi, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Enterococcus spp, Acinetobacer spp* and *Vibrio cholerae* with zone of inhibition 41.60±2.05, 36.00±2.16, 47.00±0.94, 33.60± 2.62, 43.00±0.81, 38.60±1.69, 39.00±0.81 and 44.30±2.86 respectively (table 1). The findings of antimicrobial assay clearly revealed that extracts CBLM-H and CBLM-HEt were found highly active against Gram positive strains i.e. *Bacillus subtilis* (recognized spore former bacteria), *Micrococcus luteus* (a prevailing normal flora of human skin). However, *Staphylococcus aureus* and *Staphylococcus epidermidis,* prevailing normal flora of human skin as well as dominant nosocomial pathogens, were identified as the most vulnerable to CBLM-HEt. The moderate polar fractions CBLM-Et, CBLM-EtM, CBLM-M do not show any activity against these bacteria. CBLM-H showed complete growth inhibition of *P. mirabilis and V. cholerae* at the concentration of 0.1µg/ml, whereas CBLM-HEt at 0.05µg/ml halted the growth of *S. aureus.* In antifungal activity (table 2) CBLM-H exposed inhibition against *Aspergillus niger, Aspergillus flavus*, *Trichophyton spp, Aspergillus terreus* with zone of inhibitions 17.60±1.88, 19.00±1.41, 31.00±2.41, 24.30±3.29 and two strains of yeast *Candida tropicalis* and *Candida albicans* are 25.30±2.49 and 24.00±1.63 respectively.

Table 1: Antibacterial activity of extracts and fractions of young leaves of *C. Bonduc*.

	Diameter of zone of growth inhibition of test bacterial strains, mm±SD					
	CBLM	CBLM-H	CBLM-HEt	Ethyl acetate	Methanol	Chloramphenicol
Gram positive						
Bacillus subtilis	--------	41.60±2.05	54.30±0.94	33.60±0.94	--------	39.60±1.24
Staphylococcus aureus	--------	44.30±2.86	54.00±2.16	33.60±0.94	23.60±1.24	27.30±0.47
Staphylococcus epidermidis	--------	31.00±0.81	41.30±2.05	45.30±1.15	-------	24.30±1.69
Micrococcus Luteus	--------	34.60±1.24	37.00±0.81	--------	25.60±2.62	36.00±0.81
Gram negative						
Salmonella typhi	--------	45.60±2.49	41.60±2.05	34.00±1.41	29.60±2.35	41.00±0.81
Klebsiella Spp.	--------	--------	36.00±2.16	45.30±2.05	--------	34.30±2.49
Pseudomonas areuginosa	--------	33.66±1.88	47.00±0.94	33.00±2.80	--------	16.30±1.69
E.coli	18.00±0.00	43.60±1.24	33.60±2.62	33.60±2.49	--------	35.50±0.94
Proteus mirabilis	--------	50.00±0.81	43.00±0.81	33.60± 0.94	34.00±1.41	35.00±2.49
Enterococcus Spp.	17.00±0.81	31.30±1.24	38.6±1.69	33.00±1.41	25.00±2.49	24.60±2.05
Acinetobacter Spp.	--------	37.60±3.39	39.00±0.81	33.30±1.24	--------	33.00±2.16
Vibrio cholerae	16.60±2.05	24.00±1.63	44.30±2.86	33.00±1.41	22.60±1.69	33.30±1.24

Table 2: Antifungal activity of extracts and fractions of young leaves of *C. Bonduc*.

	Diameterof zone of growth inhibition of test fungal strains, mm ±SD					
	CBLM-H	CBLM-HEt	CBLM-M	Ethyl acetate	Methanol	Nystatin
Aspergillus niger	17.60±1.88	--------	--------	19.60±1.24	--------	23.00±1.41
Aspergillus flavus	--------	24.00±0.81	--------	20.30±1.24	21.30±0.47	25.30±1.69
Trichophyton Spp.	33.66±1.88	31.00±1.24	--------	24.30±1.69	22.30±0.47	24.30±2.05
Aspergillus terreus	43.60±1.24	25.00±2.44	17.00±0.94	17.00±0.81	18.00±0.00	25.00±2.44
Peniciillium Spp.	--------	--------	--------	--------	--------	26.00±2.94
Candida tropicalis	31.33±1.24	25.30±2.49	--------	19.30±0.94	18.00±2.16	22.00±0.81
Candida albicans	24.00±1.63	18.30±1.69	--------	36.30±1.24	16.60±2.05	26.30±1.88

CBLM, CBLM-Et and CBLM-EtM did not show any zone of inhibition

Table 3: Chemical constituents of fractions identified through GC/GC-MS.

Retention time (min)	Name of identified phytochemicals	Molecular formula	Molecular mass	Class of compound	Fraction
44.362	10-Heneicosene (1)	$C_{21}H_{42}$	294	Unsaturated Hydrocarbon	CBLM-H, CBLM-Et
44.545	Sulfurous acid, butyl dodecyl ester (2) [a,b,c]	$C_{16}H_{34}O_3S$	306	Sulphurous acid	CBLM-H
44.889	Acetic acid *n*-octadecyl ester (3)[a,b,c]	$C_{20}H_{40}O_2$	312	Fatty acid esters	CBLM-H, CBLM-Et
44.970	Methyl 9,10-methylene -octadecanoate (Sterculic) (4) [a,b,c]	$C_{20}H_{38}O_2$	310	Unsaturated fatty acid esters	CBLM-H
47.086	Nonadecane (5)[a]	$C_{19}H_{40}$	268	Saturated Hydrocarbon	CBLM-H
48.894	2-Hexyl-1-decanol (6) [a,b]	$C_{16}H_{34}O$	242	Long chain alcohol	CBLM-H
48.982	7-Hexyl- eicosane (7) [a,b,c]	$C_{26}H_{54}$	366	Saturated Hydrocarbon	CBLM-H
49.802	(*Z*)-7-Hexadecenal (8) [a,b,c]	$C_{16}H_{30}O$	238	Long chain unsaturated aldehyde	CBLM-H, CBLM-Et
50.564	Heptacosane (9)	$C_{27}H_{56}$	380	Saturated Hydrocarbon	CBLM-H
50.622	Hexadecanoic acid, 2-methylpropyl ester (isobutyl palmitate) (10) [a,b,c]	$C_{20}H_{40}O_2$	312	Fatty acid esters	CBLM-H, CBLM-HEt
51.296	1,2-Benzenedicarboxylic acid, diisooctyl ester (Diisooctyl phthalate) (11) [a]	$C_{24}H_{38}O_4$	390	Plasticizer	CBLM-H, CBLM-HEt, CBLM-Et , CBLM-EtM, CBLM-M

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53.163	9-Ethyl-9-*n*-heptyloctadecane (12)[a,b,c]	$C_{27}H_{56}$	380	Saturated Hydrocarbon	CBLM-H
53.741	Terephthalic acid, di(2-ethylhexyl) ester (Bis(2-ethylhexyl) phthalate) (13)[a]	$C_{24}H_{38}O_4$	390	Plasticizer	CBLM-H
54.298	Unidentified	-	498	-	CBLM-H
55.345	3-Ethyl- 5-(2-ethylbutyl)octadecane (14) [a]	$C_{26}H_{54}$	366	Saturated hydrocarbon	CBLM-H
57.249	Unidentified	-	498	-	CBLM-H
57.309	1,1'-[1,3-Propanediylbis(oxy)]bis octadecane (15) [a]	$C_{39}H_{80}O_2$	580	Long chain ether	CBLM-H
44.369	(E)-5-Eicosene (16) [a,b]	$C_{20}H_{40}$	280	Unsaturated hydrocarbon	CBLM-HEt
44.545	2-Butyl-1-octanol (17) [a]	$C_{12}H_{26}O$	186	Long chain alcohol	CBLM-HEt
44.882	Acetic acid, *n*-octadecyl ester (n-Octadecyl ethanoate) (18) [a,b,c]	$C_{20}H_{40}O_2$	312	Fatty acid esters	CBLM-HEt
44.970	10-Nonadecenoic acid, methyl ester (19) [a,b]	$C_{20}H_{38}O_2$	310	Unsaturated fatty acid esters	CBLM-HEt, CBLM-Et
53.756	1-Monolinoleoylglycerol trimethylsilyl ether (20)[a]	$C_{27}H_{54}O_4Si_2$	498	Organic silicon	CBLM-HEt
57.769	Unidentified	-	415	-	CBLM-HEt
64.820	Unidentified	-	662	-	CBLM-HEt
05.958	Unidentified	-	174	-	CBLM-Et
06.273	Unidentified	-	132	-	CBLM-Et
07.210	Unidentified	-	56	-	CBLM-Et
23.026	2-Ethyl-2-hydroxy-1,3-dimethylcyclopentanecarboxylic acid, ethyl ester (21) [a,b,c]	$C_{12}H_{22}O_3$	214	Fatty acid esters	CBLM-Et
23.340	Unidentified	-	194	-	CBLM-Et, CBLM-M
23.685	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester (Methyl 4-hydroxycinnamate) (22) [a,b]	$C_{10}H_{10}O_3$	178	Fatty acid esters	CBLM-Et
24.746	1,3,5-Triazine-2,4-diamine, 6-hydroxy-N,N-dicyclohexyl (23) [a,b,c]	$C_{15}H_{25}N_5O$	291	Nitrogen containing compound	CBLM-Et
32.266	n-Hexadecanoic acid (24)	$C_{16}H_{32}O_2$	256	Free fatty acid	CBLM-Et
47.725	2-Hexadecanol (25)	$C_{16}H_{34}O$	242	Long chain alchol	CBLM-Et
48.989	7-Methy-*z*-tetradecen-1-ol acetate (26)	$C_{17}H_{34}O$	268	Unsaturated fatty acid ester	CBLM-Et
50.629	Hexadecanoic acid, 1-(hydroxymethyl) -1,2-ethanediyl ester (27)	$C_{35}H_{68}O_5$	568	Fatty acid esters	CBLM-Et
53.741	Unidentified	-	-	-	CBLM-Et
57.249	Unidentified	-	-	-	CBLM-Et
57.717	Unidentified	-	-	-	CBLM-Et

[a] = New from *C. bonduc*; [b] = New from genus *Caesalpinia*; [c] = New from Fabaceae.

CBLM-HEt exhibited zone of inhibition 24.00±0.81, 31.00±1.24, 25.00±2.44, 25.30±2.49 and 18.30±1.69 against *Aspergillus flavus*, *Trichophyton* spp, *Aspergillus terreus, Candida tropicalis* and *Candida albicans* respectively. CBLM-HEt found potent against *Trichophyton spp* and *Candida tropicalis* as compare to the standard drug nystatin. The fractions CBLM-Et, CBLM-EtM and CBLM-M did not show antifungal potential. Methanolic extract (CBLM) fractionated into non polar to moderately polar fractions (CBLM-H, CBLM-HEt, CBLM-Et, CBLM-EtM, CBLM-M) and subjected to GC/GC-MS analysis. Altogether twenty seven (~71%) phytochemicals were identified from different fractions (table 3, fig. 3). Individually, total identified phytochemicals in CBLM-H, CBLM-HEt, CBLM-Et, CBLM-EtM and CBLM-M were fifteen, seven, twelve, one, and one respectively. These included six fatty acid esters (3, 10, 18, 21, 22 and 27), three unsaturated fatty acid esters (4, 19 and 26), five saturated hydrocarbon (5, 7, 9, 12 and 14) and two unsaturated hydrocarbon (1 and 16), three long chain alcohol (6, 17 and 25), two plasticizer (11 and 13), a free fatty acid (24), an unsaturated long chain aldehyde (8), a long chain ether (15), sulphurous acid (2), organic silicon (20) and nitrogen containing compound (23). Twenty one (21) identified metabolites are first report from the *C. bonduc*, fourteen (14) are the first from the Caesalpinia and ten (10) from Fabaceae. Compounds 2-ethyl-2-hydroxy-1,3-dimethylcyclopentanecarboxylic acid, ethyl ester (21), 1,3,5-triazine-2,4-diamine, 6-hydroxy-N,N-dicyclohexyl

(23) are first time identified as plant metabolite using electronic mass spectral library GC-MS (NIST 20) (table 3).

DISCUSSION

In literature many identified compound from this extract have vital biological activities which may be responsible for the hepatoprotective and antimicrobial potential. Sterculic acid (4) is used as an anti-parasite drug in traditional chinese medicine and methyl esters of sterculic acid possessed parasiticidal activities against the asexual blood stage of *plasmodium falciparum* and inhibit the growth of *toxoplasma gondii* tachyzoites *in vitro* (Hao *et al*., 2016). Methyl sterculate, a cyclopropenoid fatty acid ester capable of stimulating hepatocytes in the rat to divide, was modified chemically by hydrogenation, halogenation and polymerization. These compounds were administered to rats and tested for their mitogenic effect. (Scarpelli, 1974). Hepatocytes, the major parenchymal cells in the liver which play pivotal roles in metabolism, detoxification, and protein synthesis (Zhou *et al*., 2016). Nonadecane (5) have both antioxidant and antidiabetic potentials (Senarath *et al*., 2018). Nonadecane (5) also contain insecticidal potential (Chavan. 1984). (*Z*)-7-Hexadecenal (8) exhibits antifungal, antibacterial, and antiviral activity (Devakumar, *et al*., 2017). Heptacosane (9), nonacosane, tricosane, pentadecane possess antibacterial activity (Konovalova, *et al*., 2013). Fatty acid esters possess adjuvant activity and its chain length and degree of unsaturation has noteworthy effect on it. It is observed when the acyl chain length of the fatty acid component was 16 or greater its activity increases. Isobutyl and isopropyl esters of palmitic acid adjuvant activity were superior to ethyl ester of palmitic acid. The ethyl esters of oleic (c 18: 1) and linoleic (c 18:2) acids were better than stearic (c 18: 0) (Bomford. 1981). Palmitic acid (24) and iso-butyl palmitate has been tested for hepatoprotective potential against *D*-galactosamine and results display that these compounds protected hepatocytes from being damaged by galactosamine and reduced the toxicity by 2 to 12 times with respect to 26 times by silymarin and exhibited significant hepatoprotective potential (Saxena *et al*., 2007). Bis(2-ethylhexyl)phthalate (DEHP) (13) display potent dose dependent anti-tumour activity against ehrlich ascites carcinoma cells (eac) in Swiss albino mice (Habib *et al*., 2012). 2-Ethylhexyl tetradecyl ester (13), di(2-propylpentyl) phthalate, 9-(2′,2′-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)- ethoxy] fluorine) and dodecyl 2-ethylhexyl terephthalate have individual and synergistic activity against Gram negative (*E. coli*), Gram-positive (*B. subtilis*) and (*A. flavus*) at 10, 5, 2.5 and 1.25g/mL (Osuntokun *et al*., 2019). Heptacosane, nonacosane, tricosane, pentadecane exhibited antibacterial activity (Konavaloval, *et al*., 2013). 9-Ethyl-9-*n*-heptyloctadecane (12) possesses anti-tumor, nematicidal and neurogenic activities (Jim duke, 1998). 3-Ethyl-5-(2-ethylbutyl)-octadecane (14) are effective antimicrobial and antifungal agents (Amudha *et al*., 2018). Eicosane (16) are reported as the main constituents in the Aloe vera extract responsible for high antimicrobial activity against clinical pathogens (Yogeswari *et al*., 2012). 5-Eicosene (16) has anticancer, antitumor, antidote, estrogenic, trypsin enhancer, memory enhancer, fertility enhancing and estrogenic activities (Jim duke, 1998). 1-Monolinoleoylglycerol trimethylsilyl ether (20) is the common compound in the roots of *Pistia stratiotes* and *Eichhornia crassipes* shows many biological activities such as antiarthritic, anticancer, hepatoprotective, antimicrobial, antiasthma, diuretic, antioxidant, anti-inflammatory and anti-diabetic (Tyagi *et al*., 2017) and antifouling activities (Meenakshi *et al*., 2012). *p*-Hydroxycinnamic acid possess anti-malarial and cytokine modulating activities and its derivative methyl 4-hydroxycinnamate (22) modulates inflammatory cytokine levels in malaria-infected mice through inhibition of gsk3β (Sudi *et al*., 2018) along with potential chemo-preventive activity (Trachtenberg *et al*., 2019). Antimicrobial potential are interrelated with lipophilic properties of various compound, *e.g.,* the recommended trend of antimicrobial property compared to several phytochemicals is phenols >aldehydes >ketones >alcohols >esters >hydrocarbons. Even the length of carbon chain has worthy effect on antibacterial activity (Bendiabdellah *et al*., 2013). Change in length in carbon chain, existence of double bond and even its position have prominent effects on antibacterial potency (Furtado *et al*., 2014). Many hydrocarbons have testified antimicrobial property and fatty acids along with their esters are used in food additives as antimicrobial agents (Zhang *et al*., 2013). Aliphatic alcohols possess strong-to-moderate potency against numerous bacteria and the activity depends upon the length of the carbon chain (Bendiabdellah *et al*., 2013). The presence and even the relative position of the hydroxyl group in aromatic moiety have a worthy effect on activity of that compound (Badawy *et al*., 2019).

CONCLUSION

C. bonduc is used traditionally for the treatment of a number disease and possesses large number of biological activities. In this report we have explored the biological activities and identified the chemical constituents of non-polar to moderate polar fractions. The methanolic extract (CBLM) of *C. bonduc* has a role on protection of inflammation and vacuolar degeneration of liver. Extract has a potential to be used as a hepatoprotective agent in herbal drugs. Altogether twenty seven (~71%) phytochemicals were identified from different fractions by using electronic mass spectral library GC-MS (NIST 20), of which 2-ethyl-2-hydroxy-1,3-dimethylcyclopentanecarboxylic acid, ethyl ester (21) and 1,3,5-triazine-

2,4-diamine,6-hydroxy-N,N-dicyclohexyl (23) are first time identified as plant metabolites. Literature of the identified phytochemicals exposed a pharmacological correlation of performed activities with the identified and related compounds. These phytochemical may be a responsible factor of antimicrobial and hepatoprotective activities of *C. bonduc.*

REFERENCES

Agbo EO, Bashir S, Igoli NP, Nnamonu LA, Igoli JO and Gray, AI (2015). Caesaldekarin M, A new diterpene from *Caesalpinia bonduc*. *J Nat. Prod. Res. Updates*, **1**: 1-6.

Ali H, Kabir N, Muhammad A, Shah MR, Musharraf SG, Iqbal N and Nadeem S (2014). Hautriwaic acid as one of the hepatoprotective constituent of *Dodonaea viscosa*. *Phytomed.,* **21**(2): 131-140.

Amudha P, Jayalaksmi M, Pushpabharathi N and Vanitha V (2018). Identification of bioactive components in *Enhalus acoroides* seagrass extract by gas chromatography-mass spectrometry. *Asian J. Pharma. Clin. Res.,* **11**(10): 131-137.

Badawy MEI, Marei GIK, Rabea EI and Taktak NEM (2019). Antimicrobial and antioxidant activities of hydrocarbon and oxygenated monoterpene against some food borne pathogens through *in vitro* and in silicon studies. *Pestic. Biochem. Phy.*, **158**: 185-200.

Bendiabdellah A, Dib MEA, Meliani N, Muselli A, Nassim D, Tabti B and Costa J (2012). Antibacterial activity of *Daucus crinitus* essential oils along the vegetative life of the plant. *J. Chem.,* **2013**.

Bomford R (1981). The adjuvant activity of fatty acid esters. The role of acyl chain length and degree of saturation. *Immunology*, **44**(1): 187-192.

Chavan SR (1984). Chemistry of alkanes separated from leaves of *Azadirachta indica* and their larvicidal/insecticidal activity against mosquitoes. *Schr. reihe. Gesell. Techn. Zusammenarbeit*, **161**: 59-65.

Devakumar J, Keerthana VSSS and Sudha SS (2017). Identification of bioactive compounds by gas chromatography-mass spectrometry analysis of *Syzygium jambos* (L.) collected from Western Ghats region Coimbatore, Tamil Nadu. *Asian J. Pharma. Clin. Res.*, **10**(1): 364-369.

Furtado FB, De Aquino FJ, Nascimento EA, Martins CDM, De Morais SA, Chang R and Silva CVD (2014). Seasonal variation of the chemical composition and antimicrobial and cytotoxic activities of the essential oils from *Inga laurina* (Sw.) Willd. *Molecules*, **19**(4): 4560-4577.

Gupta M, Mazumder UK, Kumar RS, Sivakumar T and Vamsi MLM (2004). Antitumor activity and antioxidant status of *Caesalpinia bonducella* against Ehrlich ascites carcinoma in Swiss albino mice. *J. Pharmacol. Sci.*, **94**(2): 177-184.

Habib MR and Karim MR (2012). Antitumour evaluation of di-(2-ethylhexyl) phthalate (DEHP) isolated from *Calotropis gigantean* L. flower/Evaluacija antitumorskog djelovanja di-(2-etilheksil)-ftalata (DEHP) izoliranog iz cvjetova *Calotropis gigantea* L. *Acta Pharmaceutica*, **62**(4): 607-615.

Hao P, Alaraj IQ, Juma'a R, Baird MS, Liu J and Liu Q (2016). Sterculic acid and its analogues are potent inhibitors of *Toxoplasma gondii*. *The Korean J. Parasitology*, **54**(2): 139-145.

Helfand SL, Werkmeister JEROME and Roder JC (1982). Chemiluminescence response of human natural killer cells. I. The relationship between target cell binding, chemiluminescence, and cytolysis. *The J. Exp. Med.*, **156**(2): 492-505.

Jim Duke, Duke's ethanobotanical and phytochemistry database. https://phytochem.nal.usda.gov/phytochem/search.

Jing W, Zhang X, Zhou H, Wang Y, Yang M, Long L and Gao H (2019). Naturally occurring cassane diterpenoids (CAs) of Caesalpinia: A systematic review of its biosynthesis, chemistry and pharmacology. *Fitoterapia*, **134**: 226-249.

Konovalova O, Gergel E and Herhel V (2013). GC-MS Analysis of bioactive components of *Shepherdia argentea* (Pursh.) Nutt. from Ukrainian Flora. *The Pharma Innov.*, **2**(6 Part A): 7.

Lin BR, Yu CJ, Chen WC, Lee HS, Chang HM, Lee YC, Chen CF (2009). Green tea extract supplement reduces D-galactosamine-induced acute liver injury by inhibition of apoptotic and proinflammatory signaling. *J. Biomed. Sci.*, **16**(1): 1-14.

Liu T, Li X, Ning Z, Qi S and Gao H (2021). Two new cassane-type diterpenoids from the seed kernels of *Caesalpinia bonduc* (Linn.) Roxb. and their anti-inflammatory activity. *Nat. Prod. Res.*, pp.1-8.

Meenakshi VK, Gomathy S, Senthamarai S, Paripooranaselvi M and Chamundeswari KP (2012). GCMS Determination of the bioactive components of *Microcosmus exasperatus* Heller, 1878. *J. Curr. Chem. Pharma. Sci.*, **2**(4): 271-276.

Osuntokun OT and Cristina GM (2019). Bio-guided isolation, chemical purification, identification, antimicrobial and synergistic efficacy of extracted essential oils from stem bark extract of *Spondias mombin* (Linn). *Int. J. Mol. Biol. Open Access*, **4**(4): 135-143.

Raghuveer R, Vijay RC, Rama RTM, Sanjeeva KA and Kashinath M (2013). Hepatoprotective activity of *Caesalpinia bonducella* leaves against carbon tetrachloride induced hepatotoxicity. *Int. J. Drug Dev. Res.,* **5**(3): 133-137.

Saxena M, Faridi U, Srivastava SK, Darokar MP, Mishra R, Pal A and Khanuja SPS (2007). A cytotoxic and hepatoprotective agent from *Withania somnifera* and biological evaluation of its ester derivatives. *Nat. Prod. Comm.*, **2**(7): 775-778.

Scarpelli DG (1974). Experimental modification of the mitogenic effect of methyl sterculate on rat liver. *Virchows. Archiv. B.*, **16**(1): 211-220.

Senarath WTPSK, Pizon JRL, Nuñeza OM, Uy, MM (2018). *In-vitro* alpha-amylase inhibitory activity, antioxidant potential, and GC-MS analysis of crepe ginger (*Costus speciosus* (J. Koenig.) Sm) leaves. *Int. J. Pharma. Sci. Res.*, **9**(11): 4741-4749.

Sindete M, Gbankoto A, Osseni R, Tossavi ND, Azonbakin S, Baba-Moussa L and Laleye A (2021). A 90-day oral toxicity study of an ethanolic root extract of *Caesalpinia bonduc* (L.) Roxb. in Wistar Rats. *Evid-Based Compl. Alt. Med.*, **2021**. (Article ID 6620026) (Open Access), https://doi.org/10.1155/ 2021/6620026

Sreelatha S, Padma PR and Umadevi M (2009). Protective effects of *Coriandrum sativum* extracts on carbon tetrachloride-induced hepatotoxicity in rats. *Food and Chem. Toxicol.*, **47**(4): 702-708.

Sudi S, Hassan WRM, Ali AH, Basir R, Embi N, Sidek HM (2018). A derivative of cinnamic acid, methyl-4-hydroxycinnamate modulates inflammatory cytokine levels in malaria-infected mice through inhibition of GSK3β. *Mal. Appl. Biol. J.,* **47**(3): 153-157.

Sundaryono A, Ekolistiono A, Astuti AP, Muryani TS, Febrianti L, Nurleli N and Nurhamidah N (2021). New candidate for traditional anti-malarial medicine from Kebiul seed (*Caesalpinia bonduc*) as a substitute for chloroquine in preclinical testing of mice. *J. Phy.: Conf. Ser.*, **1731**(1): 012021.

Trachtenberg A, Muduli S, Sidoryk K, Cybulski M and Danilenko M (2019). Synergistic cytotoxicity of methyl 4-hydroxycinnamate and carnosic acid to acute myeloid leukemia cells via calcium-dependent apoptosis induction. *Front. Pharmacol.*, **10** (Artical 507) (OPEN ACCESS).

Tyagi T and Agarwal M (2017). GC-MS analysis of invasive aquatic weed, *Pista stratiotes* L. and *Eichhornia crassipes* (Mart.) Solms. *Int. J. Curr. Pharma. Res.*, **9**(3): 111-117.

Weber LW, Boll M and Stampfl A (2003). Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. *Crit. Rev. in Toxicol.*, **33**(2): 105-136.

Yogeswari S, Ramalakshmi S, Neelavathy R and Muthumary J (2012). Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GCMS. *Global J. Pharmacol.*, **6**(2): 65-71.

Zhang X, Yang M, Song F, Zhang H and Feng F (2013). Antimicrobial activity of selected fatty acids and their derivatives. *J. Zhejiang University* (Agriculture and Life Sciences), **39**(2): 155-160.

Zhou Z, Xu MJ and Gao B (2016). Hepatocytes: A key cell type for innate immunity. *Cell. Mol. Immunol.*, **13**(3): 301-315.