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Chemical and Biological Profiling of Three Ferulic Acids Alkyl Esters Isolated from *Jatropha pandurifolia* (Family: Euphorbiaceae*)* Stem Bark

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The study's objectives include phytochemical profiling and biological (antioxidant, thrombolytic and cytotoxic) analysis of pure chemicals from *Jatropha pandurifolia* stem bark ethyl acetate extract. Five different compounds including octacosanyl cis ferulate (1), hexacosyl (E)-ferulate (2) triacontyl ferulate (3), β-sitosterol (4) and stigmasterol (5) are elucidated. Their structures determine through ^{1}HNMR analysis and comparison to published data, while three ferulic acid alkyl esters (1-3) were isolated for the first time from *J. pandurifolia*. Compounds 1, 2, and 3 all have significant thrombolytic potential with respective values of 68.92% ±1.17 (P<0.01), 66.56% ±2.35 (**P<0.01) and 70.81%±0.98 (**P<0.01) with comparison to standard streptokinase (73.6%±0.76). When compared to BHT (6.82± 0.99 ìg/ml) the IC_{50} (DPPH assay) values were 16.26±1.07 (**P<0.01), 14.12±1.23 (**P<0.01), and 13.16±1.70 µg/ml (**P<0.01). Comparing the three compounds to the reference vincristine sulphate (LC_{50}: 0.52±0.18 µg/ml), of compound 1 (1.56±0.35 µg/ml) (**P<0.01), compound 2 (1.3±0.78 µg/ml) (**P<0.01) and compound 3 (1.29±0.33 µg/ml) (**P<0.01). The results can therefore be interpreted as a concept of isolated molecules having potential for application in additional pharmaceutical research.**

Keywords: *Jatropha pandurifolia;* octacosanyl cis ferulate; hexacosyl (E)-ferulate; triacontyl ferulate; antioxidant; thrombolytic; cytotoxic activity.

The class of organic molecules known as phytochemicals, which includes tannins, sugars, triterpenoids, alkaloids, flavonoids, steroids, and others, is rife in medicinal plants (plant chemicals). These phytochemicals are fantastic for offering health advantages and acting as a protection, avoiding a number of diseases [1].

Excessive generation of reactive oxygen species (ROS) leads to oxidative stress [2] that in turn trigger several diseases such in inflammation, hypertension, atherosclerosis, AIDS, DM and cancer etc. Antioxidative enzymes such as peroxidase, superoxide dismutase (SOD) is concealed by the over production of free radicals

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that consequences cellular mutation and damage [3]. The brine shrimp bioassay is linked to cytotoxic act in living tissue and represents pesticidal activity [4,5]. The mechanism of a thrombolytic drug is to liquefy blood clot (thrombus) in acutely occluded coronary arteries, pulmonary embolism, atrial fibrillation, thrombosis and prosthetic heart valves [6] that ensures sufficient blood supply to myocardium and to develop prospects with blood loss reduction. Market available thrombolytic agents like urokinase, alteplase, streptokinase, anistreplase, tissue-type plasminogen activator (tPA) offer some restrictions like side effects with associated bleeding history [7,8]. Due to the presence of phytochemical elements, medicinal plants are essential in the prevention of various diseases, and their use and promotion fit into all current prevention efforts.

Jatropha (Family: Euphorbiaceae, a castor family) having 175 species of succulent, caudiciform, herbaceous perennials and woody plants including *Jatropha pandurifolia (ýJatropha integerrima)* that is a tiny, evergreen tree or shrub with lustrous leaves with star-shaped red, pink, or vermilion blooms and also known as peregrine or spicy jatropha [9]. In Asia, Latin America, and Africa, several plant parts including the leaves, stem, bark, roots, and oil, have long been used for a variety of ailments as styptic agent, emetic agent, antidote [10,11], purgative [12], in the treatment of warts, toothaches, ringworm, gastric emptying, gum bleeding, rheumatic pain and skin diseases [13,14]. Numerous phytochemicals holding potential medicinal norms were reported from this plant including alkaloid (jatrophine, jatropham and curcin) [15], diterpene (jatrophone, jatrophatrione, jatropholone A–B) [16,17], sitosterol, glycoside, lignin, tannin, saponin [18], fatty acids (palmitic, oleic, and linoleic acids), carotenoid, flavonoid etc [19,20]. This plant has been linked to antiinflammatory, antituberculosis, antiplasmodial, cytotoxicity [21,22], anticancer [23,24], antimicrobial [25-27], antifungal antioxidant potentials [28] according to previous studies. Following several chromatographic methods structure elucidations was carried out by HNMR spectroscopy. The chemical investigation revealed very first isolation of three ferulic acid alkyl esters from this species. β-sitosterol and stigmasterol were also isolated concurrently. In the current study the obtained pure three ferulic acid alkyl esters were subjected to antioxidant, thrombolytic and cytotoxic investigation.

MATERIALS AND METHODS

Overall experimental measures

Whilst performing VLC (vacuum liquid chromatography) fine VLC-grade silica like Kiesel gel 60H was used in the column. With the aid of dichloromethane, ethyl acetate, and methanol, the elution was carried out while gradually increasing polarity. An appropriate solvent system was used to elute TLC plates that are pre coated with Silica gel 60, F254. The TLC plates were sprayed with vanillin-H_2SO_4, heated for 5-10 minutes at 110°C, and UV light at short wavelength (254) and long wavelength (366) nm were used to visualize the spots. NMR spectra were transcribed in $CDCl_3$ on a 400 MHz Ultra shield NMR Spectrophotometer.

In August 2019, *J. pandurifolia* stem barks were collected from premises of University of Dhaka, Bangladesh. The plant was identified in National Herbarium, Dhaka and an identification no. was preserved (DACB Accession No. 65545). The air dried and powdered stem-barks of *J. pandurifolia* (3.0 kg) in ethyl acetate over the period of 15 days and filtered through a cotton pad then by Whatman filter paper. The extract was then evaporated using a Buchii rotary evaporator to be concentrated under reduced pressure.

Extraction and Isolation

An aliquot part of the crude ethyl acetate extract (40g) was partitioned by VLC technique in increasing order of polarity to provoke adsorption and seperation like a normal-phase column chromatography. Used solvents were petroleum ether, ethyl acetate and ethanol in sequence. VLC fractions were numbered from 1 to 41. White crystals were appeared in fraction VLC 10, 13 and 25. The crystals were washed with n-hexane, collected separately and were purified by PTLC (preparative TLC) with a convenient solvent system (1% ethyl acetate in toluene). By recrystallization technique JP-10 (Compound 1, colorless crystals, 5.0 mg, R_f value 0.65), JP-13 (Compound 2, colorless crystals, 10.0 mg, R_f value 0.45) and JP-25 (Compound 3, colorless crystals, 4.0 mg, R_f value 0.50), JP-5 (Compound 4, clear liquid, 3 mg, R_f value 0.60), JP-6 (Compound 5, clear liquid, 2 mg, R_f value 0.50) were yielded.

Spectral data of the compounds (C1-3)

Compound 01 (JP-10)

^{1}HNMR (400 MHz, $CDCl_3$); δ 5.82 d (J = 12 Hz, doublet,) (H-2), δ6.79 d (J = 12 Hz, doublet) (H-3), δ7.77 s (H-5), δ6.88 (J = 8 Hz, doublet) (H-8), δ7.10 (J = 8 Hz, doublet) (H-9), δ4.11 (J = Hz, triplet, 2H) (H-1'), δ1.25-1.34 (multiplet, 50H) (H 3'-27'), δ1.64 (multiplet, 2H) (H-2'), δ0.88 (6.5) (triplet, 3H) (H-28'), δ3.93 (singlet, 3H) and δ5.83 (doublet) for OCH_3 and OH-7 groups.

Compound 02 (JP-13)

^{1}HNMR (400 MHz, $CDCl_3$); δ 6.29 (J = 16 Hz, doublet,) (H-2), δ7.61 d (J = 16 Hz, doublet) (H-3), δ7.03 (singlet, H-5), δ6.91 (J = 8 Hz, doublet) (H-8), δ7.07 (J = 8.1 Hz, doublet of doublets) (H-9), δ 4.18(triplet, 2H) (-1'), δ1.25-1.39 (multiplet, 46H) (H 3'-25'), δ1.68 (multiplet, 2H) (H-2'), δ0.88 (6.5) (triplet, 3H) (H-26'), δ3.92 (singlet, 3H) and δ5.93 (singlet with broad signal) for OCH_3 and OH-7 groups.

Compound 03 (JP-25)

^{1}HNMR(400 MHz, $CDCl_3$); δ 6.29 (J = 16 Hz, doublet,) (H-2), δ7.61 d (J = 16 Hz, doublet) (H-3), δ7.03 (singlet, H-5), δ6.91 (J = 8 Hz, doublet) (H-8), δ7.07 (J = 8.1 Hz, doublet of doublets) (H-9), δ4.18 (triplet, 2H) (H-1'), δ1.25-1.41 (multiplet, 46H) (H 3'-29'), δ1.68 (multiplet, 2H) (H-2'), δ0.88 (6.5) (triplet, 3H) (H-30'), δ3.93 (singlet, 3H) and δ5.85 (singlet with broad signal) for OCH_3 and OH-7 groups.

Structure elucidation of C-4 and C-5 were also performed by ^{1}HNMR analysis and compared spectral data were compared to the reference data**.**

Protocol for biological investigation

Isolated pure compounds were taken for in vitro investigations according to established methods.

Thrombolytic activity

The percentage of human blood clot lysis was estimated [29] in comparison to Streptokinase (100ìl equivalent to 30,000 I.U) as positive control and distilled water (100 µl) as negative control. The calculation as follows:

% of thrombolytic activity = [(wt. of clot after treatment / wt. of clot before treatment) \ Antioxidant activity (DPPH free radical scavenging assay) × 100]

According to Brand-Williams *et al.* [30] DPPH assay was carried out in presence of different concentration by serial dilution technique. Butylated hydroxyl toluene (BHT) and methanolic DPPH solution were used as positive control and negative control accordingly. IC_{50} values were calculated from the regression equation by plotting concentrations vs percentage of scavenging the free radicals.

$$I\% = 1- \{A_1 / A_0\} \times 100$$

$A_{0:}$ absorbance of the control and A_1: absorbance of test samples/standard.

The experiment was repeated in triplicate at each concentration.

Brine shrimp lethality bioassay

The cytotoxic test was anticipated by brine shrimp lethality bioassay [31] of pure compounds at different concentrations. The percentage of mortality was calculated and plotted against LogC Then lethal concentrations (LC_{50}) were calculated to measure cytotoxic activity. Saline water and vincristine sulphate (VS) were employed as negative and positive controls, respectively.

% mortality = (no. of dead nauplii/initial total no. of live nauplli) × 100

Statistical Analysis

The results of every experiment were shown as mean SEM. Using SPSS 20 software, one-way analysis of variance (ANOVA) was used to compare the study groups to one another. A p value of 0.05 or less was considered statistically significant.

RESULTS AND DISCUSSION

Structure Elucidation

Modern chromatographic purification and separation of the ethyl acetate-stem bark extract of *J. pandurifolia* provided a total of three compounds (1-3), the structures were elucidated by ^{1}H NMR analysis.

Compound 01 (JP-10)

Colorless crystal. Black and bluish spots were observed under the both 254 nm and 356 nm UV light accordingly on TLC and deep purple spot

with vanillin-H_2SO_4 and heating for 1 min at 110^0C. Three aromatic protons were seen in the ^{1}HNMR spectrum at δ 7.77 s, δ6.88 d (*J* = 8 Hz) and δ7.10 d (*J* = 8 Hz) assignable to H-5, H-8 and H-9 of phenyl ring respectively. Protons at position 2 and 3 having doublets at δ5.82 (*J* = 12 Hz) and δ6.79 (*J* = 12 Hz) respectively are close to the carboxylic group. A triplet was appeared at δ 4.11 representing a methylene group. The spectrum also revealed two multiplets at δ1.25-1.34(50H) and δ1.64 for $-CH_2$ group and a triplet at δ0.88 for a $-CH_3$ group. A broad singlet (δ3.93) and doublet (δ5.83) indicate

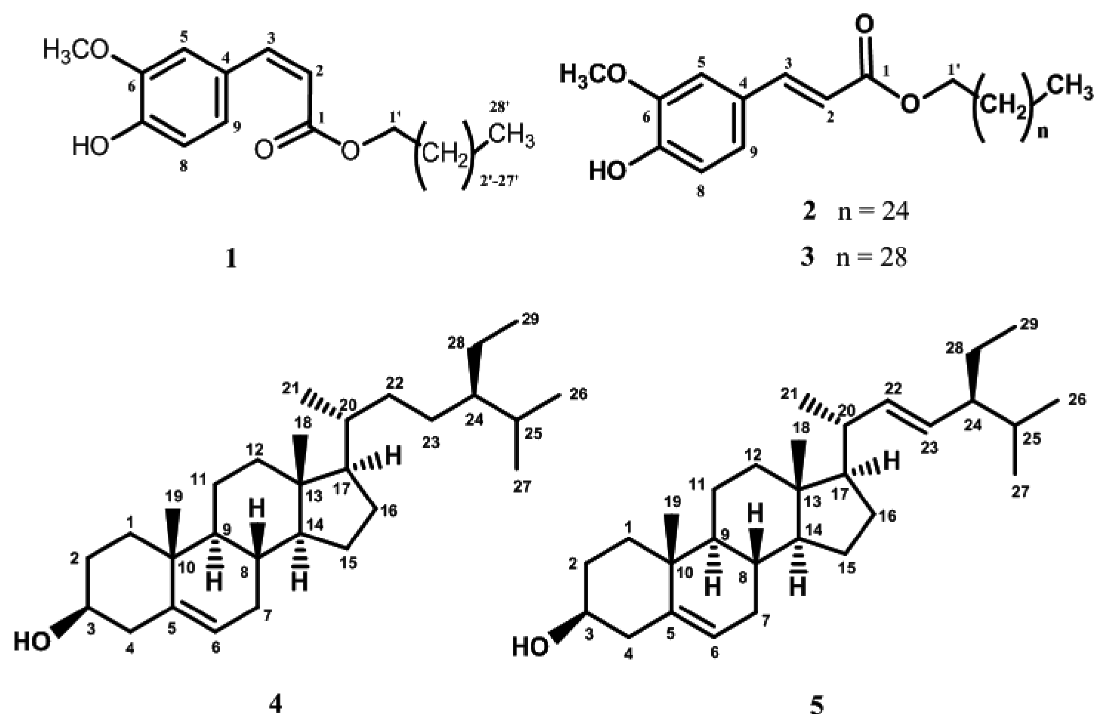


Fig. 1. Compounds (1-5) isolated from EASBJP

Table 1. ^{1}H NMR data for compounds (1-3) (400 MHz in CDCl3, δ in ppm, J in Hz)

Position	JP-10 δH	Position	JP-13 δH	Position	JP-25 δH
1	—-	1	—-	1	—-
2	5.82 d (*J* = 12 Hz)	2	6.29 d (*J* = 16 Hz)	2	6.29 d (*J* = 16 Hz)
3	6.79 d (*J* = 12 Hz)	3	7.61 d (*J* = 16 Hz)	3	7.61 d (*J* = 16 Hz)
4	—	4	—	4	—
5	7.77 br s	5	7.03 br s	5	7.03 br s
6	—-	6	—-	6	—-
7	—	7	—	7	—
8	6.88 d *J* = 8 Hz)	8	6.91 d (*J* = 8 Hz)	8	6.91 d (*J* = 8 Hz)
9	7.10 d (*J* = 8 Hz)	9	7.07 dd (*J* = 8.1 Hz)	9	7.07 dd (*J* = 8.1 Hz)
1’	4.11 t (2H, *J* = 6.5 Hz)	1’	4.18 t (2H, *J* = 6.5 Hz)	1’	4.18 t (2H, *J* = 6.5 Hz)
2’	1.64 2H m	2’	1.68 2H m	2’	1.68 2H m
3’-27’	1.25-1.34 (50H) m	3’-25’	1.25-1.39 (46H) m	3’-29’	1.25-1.41 (54H) m
28’	0.88 t (3H, *J* = 6.5 Hz)	26’	0.88 t (3H, *J* = 6.5 Hz)	30’	0.87 t (3H, *J* = 6.5 Hz)
OCH_3	3.93 3H s	OCH_3	3.92 3H s	OCH_3	3.93 3H s
OH-7	5.83 s	OH-7	5.93 br s	OH-7	5.85 s

methoxy and hydroxyl groups consecutively on the phenyl ring. Compared ^{1}HNMR data with those of compound-1 was determined to be octacosanyl cis ferulate [32].

Compound 02 (JP-13)

Colourless crystal. Black and bluish spots were observed under the both 254 nm and 356 nm UV light accordingly on TLC and deep purple spot with vanillin-H_2SO_4 and heating for 1 min at 110⁰C. Three aromatic protons were seen in the ^{1}HNMR spectrum at δ7.03 s, 6.91 d (*J* = 8 Hz) and δ7.07 dd (*J* = 8.1 Hz) assignable to H-5, H-8 and H-9 of phenyl ring respectively. Protons at position 2 and 3 having doublets at ä6.29 (*J* = 16 Hz) and 7.61 (*J* = 16 Hz) respectively are close to the carboxylic group. A triplet was appeared at δ 4.18 representing a methylene group. The spectrum also revealed two multiplets at δ1.25-1.39 (46H) and δ 1.68 for $-CH_2$ group and a triplet at δ 0.88 for a $-CH_3$ group. A singlet (δ3.92) and broad singlet (δ 5.93) indicate methoxy and hydroxyl groups consecutively on the phenyl ring. Compared NMR data with those of compound-2 was determined to be hexacosyl (E)-ferulate [33].

Compound 03 (JP-25)

Colourless crystal. Black and bluish spots were observed under the both 254 nm and 356 nm UV light accordingly on TLC and deep purple spot with vanillin-H_2SO_4 and heating for 1 min at 110⁰C. The ^{1}HNMR spectrum displayed three aromatic protons at δ 7.03 s, 6.91 d (*J* = 8 Hz) and δ7.07 dd (*J* = 8.1 Hz) assignable to H-5, H-8 and H-9 of phenyl ring respectively. Protons at position 2 and 3 having doublets at δ 6.29 (*J* = 16 Hz) and δ7.61 (*J* = 16 Hz) respectively are close to the carboxylic group. A triplet was appeared at δ 4.18 representing a methylene group. The spectrum also revealed two multiplets at δ1.25-1.41 (54H) and δ1.68 for $-CH_2$ group and a triplet at δ 0.88 for a $-CH_3$ group. A singlet (δ 3.93) and broad singlet (δ 5.85) indicate methoxy and hydroxyl groups consecutively on the phenyl ring. Compared NMR data with those

Table 2. Thrombolytic activity of pure compounds (1-3) isolated from EASBJP

Sample ID	% of clot lysis
Compound 1	68.92** ± 1.17
Compound 2	66.56**± 2.35
Compound 3	70.81**± 0.98
Streptokinase (STK)	73.6 ± 0.76
Negative control (water)	5.03± 0.70

Values are expressed as mean ± SD (n = 3); Significance level among different groups at P <0.05 (*P<0.05; **P<0.01, ***P<0.001); Test groups were compared to standard group; EASBJP represents ethyl acetate stem bark of *J. pandurifolia*

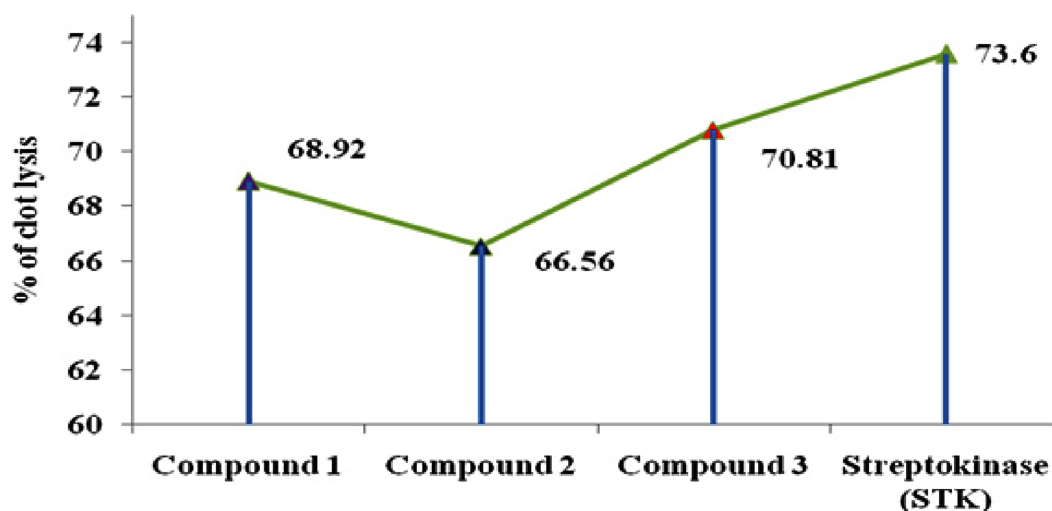


Fig. 2. Thrombolytic activity of pure compounds **(1-3)** isolated from EASBJP

Compound **1**, compound **2** and compound **3** showed significant clot rupture activity valued as 68.92% ± 1.17 (**P<0.01), 66.56% ± 2.35 (**P<0.01), and 70.81% ± 0.98 (**P<0.01) accordingly in comparison to Streptokinase (73.6% ± 0.76) (Tab: 02, Fig: 02).

of compound-3 was determined to be triacontyl ferulate [34].

Biological investigations of *J. pandurifolia*

The isolated pure crystals confirmed various degrees of secondary metabolites when subjected to thrombolytic, antioxidant and brine shrimp cytotoxic activities.

Thrombolytic activity

Tissue plasminogen activator (t-PA), streptokinase, alteplase, reteplase, tenecteplase and urokinase etc. are commonly prescribed thrombolytic medication for the treatment of various CVD, myocardial infarction, cerebral thrombosis etc. Several secondary metabolites such as alkaloids, tannins, flavonoids, saponins exert thrombolytic properties as a natural supply [35].

Antioxidant activity

Other than superoxide dismutase, catalase enzymes or glutathione transferase diverse phytochemicals like polyphenolic compounds such as, flavonoids, phenolics, tocopherols, tannins etc. are to blame for inhibiting oxidation that may cause cell damage in living organism [36]. The present study depicted IC_{50} value 6.82± 0.99 µg/ml for butylated hydroxy toluene (BHT).

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is a quick and dynamic technique to compute the cytotoxicity of any compounds that might be noxious to health [38]. The pure compounds showed significant brine shrimp larvicidal activity in comparison to positive control (Vincristine sulphate, LC_{50}: 0.52 ± 0.18). LC_{50} were measured for compound 1, 2 and 3 were 1.56 ± 0.35 (**P<0.01), 1.3 ±0.78 (**P<0.01), 1.29 ± 0.33 (**P<0.01) (µg/ml) indication less toxicity compared to the positive control. The results point toward the presence of relevant influential phytotoxic secondary metabolites that may be deleterious to living organisms.

Earlier research has reported the cytotoxic and antimicrobial activity from the n- hexane stem bark extract of this plant [22]. Antioxidant activity was reported from different extracts of root and leaf of *J. gaumeri* [39], *J.Unicostata* [40]*, J. macrantha* [41]. Investigations of *J. curcas, J. integerrima, J. multifida, J. chevalieri, J. gossypiifolia, J. podagrica* and *J. pohliana* have exerted cytotoxic activity towards toward melanoma cells, human cancer of the nasopharynx, artemia and other cell line analysis [42,43] whereas aqueous and ethanolic

Table 3. Antioxidant activity of pure compounds (1-3) isolated from EASBJP

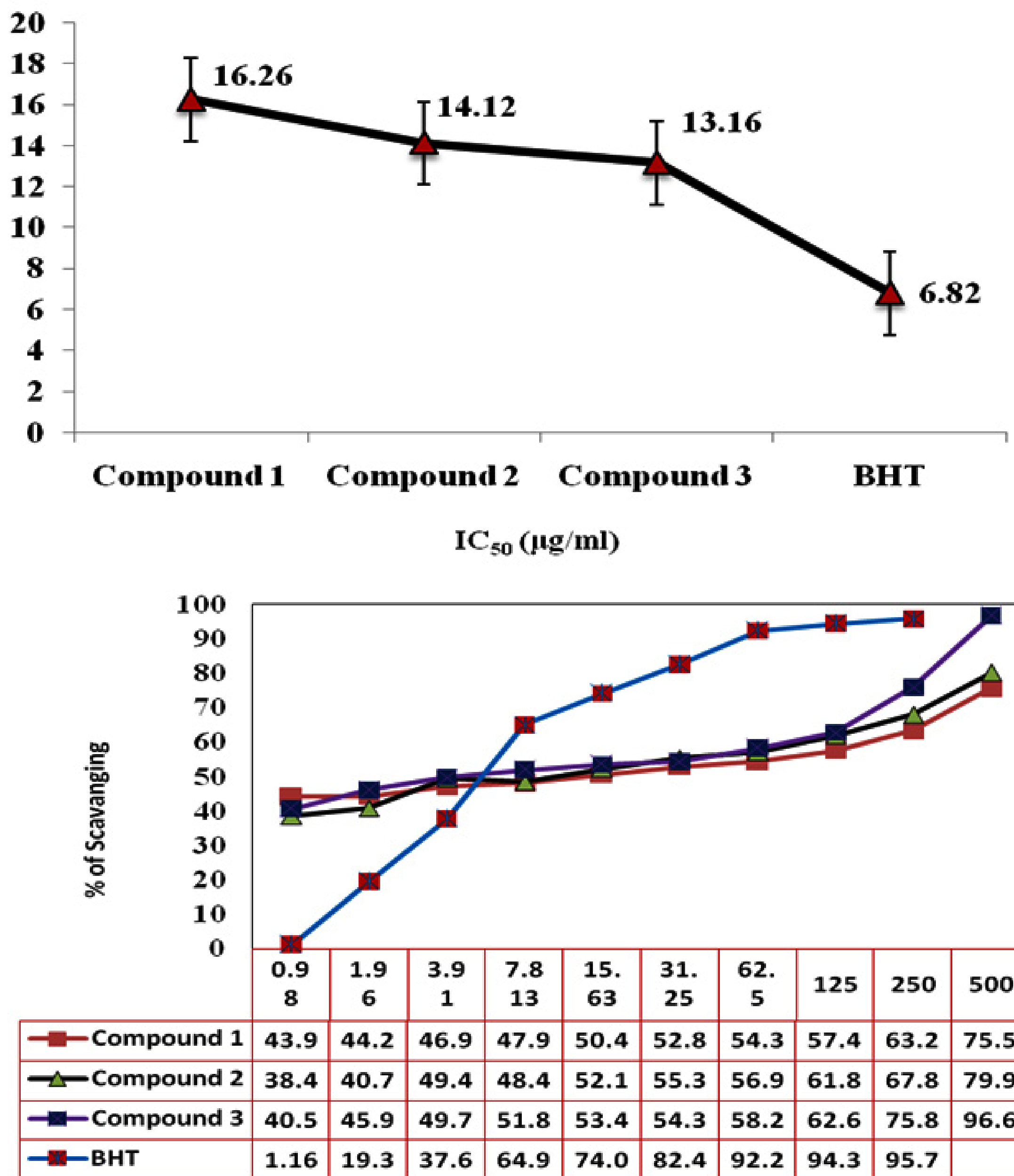
Sample ID	DPPH scavenging activity (IC_{50}, ìg/ml)	Regression equation
Compound 1	16.26** ± 1.07	y = 0.019x + 0.191, R² = 0.853
Compound 2	14.12** ± 1.23	y = 0.025x + 0.147, R² = 0.928
Compound 3	13.16** ± 1.7	y = 0.031x + 0.092, R² = 0.800

Values are expressed as mean ± SD (n = 3); Significance level among different groups at P ≤0.05 (*P<0.05; **P<0.01, ***P<0.001); Test groups were compared to standard group; EASBJP represents ethyl acetate stem bark of *J. pandurifolia*

Table 4. Cytotoxic activity of pure compounds (1-3) isolated from EASBJP

Sample ID	Cytotoxicity (LC_{50} ìg/ml)	Regression equation
Compound 1	1.56** ± 0.35	y = 29.90x + 3.478, R² = 0.951
Compound 2	1.3** ±0.78	y = 22.35x + 21.13, R² = 0.944
Compound 3	1.29** ±0.33	y = 26.27x + 16.16, R² = 0.972

Values are expressed as mean ± SD (n = 3); Significance level among different groups at P ≤0.05 (*P<0.05; **P<0.01, ***P<0.001); Test groups were compared to standard group; EASBJP represents ethyl acetate stem bark of *J. pandurifolia*



	0.98	1.96	3.91	7.813	15.63	31.25	62.5	125	250	500
Compound 1	43.9	44.2	46.9	47.9	50.4	52.8	54.3	57.4	63.2	75.5
Compound 2	38.4	40.7	49.4	48.4	52.1	55.3	56.9	61.8	67.8	79.9
Compound 3	40.5	45.9	49.7	51.8	53.4	54.3	58.2	62.6	75.8	96.6
BHT	1.16	19.3	37.6	64.9	74.0	82.4	92.2	94.3	95.7	

Fig. 3. Comparative Antioxidant activity of pure compounds (1-3) isolated from EASBJP Compound 1, 2 & 3 exerted DPPH scavenging property with IC_{50} valued 16.26 ± 1.07 (**P<0.01), 14.12 ± 1.23 (**P<0.01) & 13.16 ± 1.70 µg/ml (**P<0.01) accordingly that projects on mild to moderate antioxidative potential of the isolated compounds that could be an innate reserve of polyphenolic compounds. Turkmen et al. (2006) hypothesized that solvent polarity may be a factor in the effectiveness of various extracts at scavenging free radicals [37].

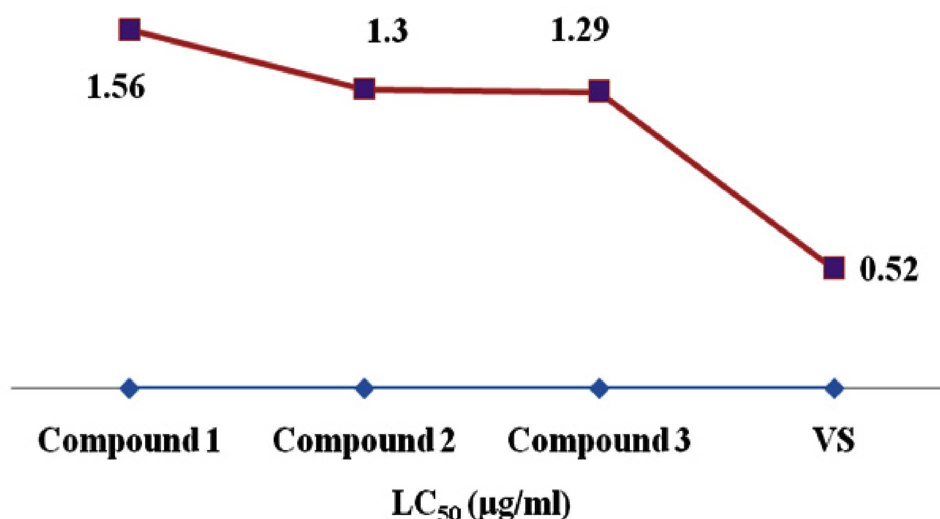


Fig. 4. Cytotoxic activity of pure compounds (1-3) isolated from EASBJP

leaf extract *J. macrantha* showed toxicity on brine shrimp (Artemia sp.) model [44]. Latex and leaf of *J. curcas* [45] and *J. gossypiifolia* [46] were turned up as blood thinner with probable estimation in clotting time reduction which could be correlated to thrombolytic property of the current plant. Biological investigations of the pure compounds from this plant strongly prove the abundance of ethno pharmacological activity. Further investigations of the isolated compounds are required specially, biological and molecular docking studies for unveiling probable activities.

CONCLUSION

The biological investigation found in this research justifies the pharmacological activity of this plant for curing miscellaneous malady. Different chain length of the ester increases the antioxidant potential of ferulic acids. Mild to strong thrombolytic, antioxidant and cytotoxic activities were unveiled by the pure compounds of ethyl acetate fraction that would be remarkable investigation of unrevealed ethno-pharmacological potency. The present study suggests exploring extensive research on this plant to discover many potent bioactive molecules.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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