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Antimicrobial and Cytotoxic Activity of Di-(2-ethylhexyl) Phthalate and Anhydrosophoradiol-3-acetate Isolated from *Calotropis gigantea* (Linn.) Flower

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A phytochemical study on the flowe r of *Calotropis gigantea* (Linn.) using silica gel column chromatography and preparative thin layer chromatography, led to the first time isolation of Di-(2-ethylhexyl) phthalate (compound 1) and anhydrosophoradiol-3-acetate (compound 2). The structures of these compounds were confirmed by spectroscopic analyses (IR, HRTOFMS and NMR). The antibacterial and antifungal activities of ethyl acetate extract, compound 1 and compound 2 were measured using the disc diffusion method. Ethyl acetate extract and compound 1 presented better results than compound 2. The minimum inhibitory concentrations (MICs) of the extract and compounds were found to be in the range of 16~128 $\mu g/ml$. The cytotoxicity (LC_{50}) against brine shrimp nauplii (*Artemia salina*) were also evaluated and found to be 14.61 $\mu g/ml$ for ethyl acetate, 9.19 $\mu g/ml$ for compound 1 and 15.55 $\mu g/ml$ for compound 2.

KEYWORDS : Anhydrosophoradiol-3-acetate, Antimicrobial, *Calotropis gigantea* and Di-(2-ethylhexyl) phthalate

The emergence of human pathogenic microorganisms that are resistant to major classes of antibiotics has been increased in recent years, due to the indiscriminate use of antimicrobial drugs (Karaman *et al*., 2003). But this has caused many clinical problems in the treatment of infectious diseases and the antibiotics commonly used are sometimes associated with adverse effects on the host, which include hypersensitivity, allergic reaction and immunosuppression (Mukherjee *et al*., 2002). Plants are known to produce some chemicals, which are naturally toxic to bacteria and fungi (Basile *et al*., 1999). Therefore, research for development of new antimicrobial agents from plants is an urgent need.

Calotropis gigantea L. (Asclepiadaceae) is a laticiferous shrub widely distributed in Bangladesh, India, Burma, Pakistan and sub Himalayan tract (Kartikar and Basu, 1994). The roots and leaves of *Calotropis gigantea* are used traditionally for treatment of abdominal tumors, boils, syphilis, leprosy, skin diseases, piles, wounds, rheumatism, insect-bites, ulceration and elephantiasis (Ghani, 2003). Various parts of this plant have been reported to possess multiple therapeutic properties like anti-inflammatory, analgesic, anticonvulsant, anxiolytic, sedative, antidiarrhoeal and antipyretic (Adak and Gupta, 2006; Argal and Pathak, 2006; Chitme *et al*., 2004; Chitme *et al*., 2005). A literature review showed that *Calotropis gigantea* contained cardenolide glycosides (Mueen *et al*., 2005; Lhinhatrakool and Sutthivaiyakit, 2006; Kiuchi *et al*., 1998), pregnanes (Kitagawa *et al*., 1992; Shibuya *et al*., 1992), a nonprotein amino acid (Pari *et al*., 1998), terpenes (Gupta and Ali, 2000; Thakur *et al*., 1984; Anjaneyulu and Row, 1968), Flavonoids (Sen *et al*., 1992) and steroids (Habib *et al*., 2007; Basu and Nath, 1934). Powdered flowers of *Calotropis gigantea*, in small doses, are also useful in the treatment of colds, cough, asthma, catarrh, indigestion, inflammatory diseases and loss of appetite (Ghani, 2003). Stomachic, digestive and analgesic properties of *Calotropis gigantea* flowers have been reported in literature (Kartikar and Basu, 1994; Pathak and Argal, 2007). People in Indian-subcontinent including Bangladesh used *Calotropis gigantea* flowers as a traditional flock medicine in small pox, muscular pain, convulsions, scabies, and a number of ailments (Mueen *et al*., 2005; Ghani, 2003). The present study was undertaken to investigate the *in vitro* antimicrobial activity of isolated compounds from *Calotropis gigantea* flower against some pathogenic bacteria and fungi as well as cytotoxic activity against brine shrimp nauplii.

Materials and Methods

General methods. IR-spectra were taken on FTIR-8900 spectrophotometer (Shimadzu Kyoto, Japan) and High Resolution TOF Mass Spectra were obtained using a Waters LCT Premier mass spectrometer (UK) coupled with a Waters AQUITY HPLC system, with data acquisition achieved using MassLynx software, version 4.0. GCMS-QP2010S (Shimadzu Kyoto, Japan) spectrometer was used for taking GC-MS. NMR spectra were recorded on Bruker 400 MHz FT spectrometer (DPX-400, Switzarland). All the spectra were taken in Analytical Research Division, Bangladesh Council of Scientific and Industrial

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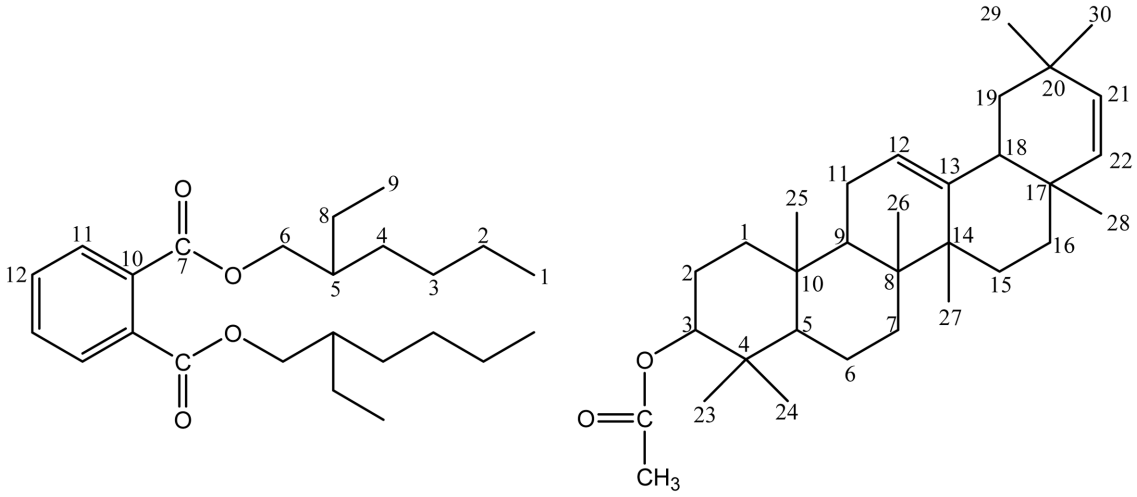
Plant material. The flowers (flower's petal) of *Calotropis gigantea* were collected in April, 2008 from the relevant area (Meherchandi) of Rajshahi University campus and authenticated by Professor A. T. M. Naderuzzaman, Department of Botany, University of Rajshahi. A voucher specimen (No. 1A. Alam, Collection date 15.08.2004) was preserved in the Department of Botany, University of Rajshahi, Bangladesh.

Extraction and isolation. The shed-dried powdered flower (1.0 kg) of *Calotropis gigantea* was extracted with ethyl acetate (1.5 *l*) at room temperature. The solvent was completely removed by rotary vacuum evaporator from the crude extract to yield a residue of 38 g. Then crude ethyl acetate extract (10 g) was applied on silica gel (60~120 mesh) chromatography using n-hexane with a gradient of ethyl acetate up to 100% and followed by chloroform. Sixty four (64) fractions were collected. Among these fractions, fraction 21~30 afforded compound **2** as white crystals (75 mg). Fractions 40~48 were subjected to preparative TLC (n-hexane-methanol: 20 : 0.1) to afford the pure compound **1** (98 mg) as colorless oily liquid. The purity of the isolated compounds was checked on TLC plates.

Microorganisms. Four Gram positive (*Staphylococcus aureus* ATCC25923, *Bacillus subtilis* QL40, *Bacillus megaterium* QL38 and *Sarcina lutea* QL166), four Gram negative (*Escherichia coli* ATCC27853, *Shigella sonnei* C182, *Shigella shiga* C180 and *Shigella dysenteriae* ATCC26131) pathogenic bacterial strains and four fungal strains (*Aspergillus niger* ATCC235561, *Aspergillus flavus* ATCC10558, *Aspergillus fumigatus* ATCC10231, and *Fusarium* sp ATCC56390) were collected from the Institute of Biological Science (IBS), University of Rajshahi, Bangladesh.

Antibacterial and antifungal study. The ethyl acetate extract, compound **1** and compound **2** were tested separately for antibacterial and antifungal activity by disc diffusion assay method (Rois *et al.*, 1988). *Kanamycin* disc (30 μg/disc) and *Nystatin* disc (100 μg/disc) were used as positive antibacterial and antifungal control, respectively. Blank disc impregnated with the respective solvent was used as negative control. The antibacterial activity of each sample was tested against each bacterium at concentrations of 30 μg/disc, 60 μg/disc and 90 μg/disc. For antifungal screening, each sample was tested at concentrations of 100 μg/disc, 200 μg/disc and 400 μg/disc. Antibacterial assay plates were incubated at 37 ± 1°C for 24 h and antifungal assay plates were incubated at 37 ± 1°C for 48 h. Each experiment was carried out in triplicates, and diameter of the zone of inhibition surrounding each disc was recorded. The minimum inhibitory concentration (MIC) for the samples having antimicrobial activity, were also determined by serial dilution technique (Reiner, 1982).

Brine shrimp lethality bioassay. The experiment was carried out using the method described by Meyer (Meyer *et al.*, 1982). In brief, *Artemia salina* Leach (brine shrimp eggs) was allowed to hatch and mature as nauplii (Larvae) in seawater for 48 h at 25°C. Serially diluted test solutions (80 μl in DMSO from a stock solution of 5 mg/*ml* DMSO) were added to the seawater (5 *ml*), containing 10 nauplii. After incubation for 24 h at 25°C, the number of survivors was counted. The LC_{50} (50% lethal concentration, $\mu g/ml$) was determined from triplicate experi-



Di-(2-ethylhexyl) phthalate
(Compound **1**)

Anhydrosophoradiol-3-acetate
(Compound **2**)

Fig. 1. Structures of compounds **1** and **2**.

ments. Ampicillin trihydrate was used as positive control.

Results and Discussion

Isolated and purified compounds **1** and **2** were characterized by IR, Mass and NMR spectral data. Molecular formula for compound **1** was deduced as $C_{24}H_{38}O_4$ through EI-MS which showed the molecular ion (M^+) peak at *m/z* 390.3617 (calcd for $C_{24}H_{38}O_4$). The presence of a phthalate was inferred from the EI-MS peaks at *m/z* 167 (48) and *m/z* 149 (100). The IR spectrum revealed a carbonyl band observed at 1739 cm^{-1} and strong C-O bands in the range 1047~1250 cm^{-1}. The aromatic signals between 6.96 (dd) and 7.11 (dd) ppm on the ^{1}H-NMR spectrum of compound **1** have reasonable coupling constants for protons at the ortho-substituted ring. Signal at 4.15 (m) ppm is assigned to a methylene group geminal to the ester alcohol group. The ^{13}C-NMR spectrum of compound **1**, confirming the symmetry of the molecule, exhibited the expected 12 carbon resonances (Table 1) assigned by DEPT experiment to two quaternary, three methine and five methylene carbons with two methyl groups. By comparison of ^{1}H and ^{13}C-NMR data to those published in literature (Rao *et al.*, 2000; Amade *et al.*, 1994), compound **1** was identified as Di-(2-ethylhexyl) phthalate (DEHP). DEHP (compound **1**) is a well known synthetic plasticizer, already reported to be present in *Alchornea cordifolia* (Mavar-Manga *et al.*, 2008), *Aloe vera* (Lee *et al.*, 2000), *Euphorbia cyparissias* and *Euphorbia seguieriana* (Toth-Soma *et al.*, 1993) and may have a taxonomic significance. The effective presence of compound **1** in flowers, not as a contaminant from solvents, was further confirmed by GC-MS analysis. The plant flowers were not conserved in plastic bags, so these could be discounted as a source of DEHP. The present study could not determine if DEHP is synthesized by the plant, absorbed by the roots or adsorbed from external atmosphere, but this compound, whatever its origin, appears likely to be

Table 1. ^{1}H- and ^{13}C-NMR spectral data of Compound **1** and **2**

Carbon no.	Compound **1**		Compound **2**	
	δ_C	δ_H	δ_C	δ_H
1	14.11	0.84 (3H, t, J = 5.3 Hz)	28.14	2.01 (2H, d, J = 11.5 Hz)
2	24.80	1.23~1.40, m	23.41	1.67 (2H, s)
3	22.68	1.23~1.40, m	81.0	4.50 (1H, t, J = 11.6 Hz)
4	29.50	1.23~1.40, m	28.10	
5	40.76	2.606, m	55.32	1.67 (1H, s)
6	65.21	4.15, m	18.29	1.66 (2H, s)
7	171.10		29.72	1.65 (1H, s)
8	29.67	2.30 (2H, dq, J = 4.3 Hz)	39.69	
9	20.79	0.93 (3H, t, J = 4.3 Hz)	47.70	2.00 (1H, s)
10	124.75		28.77	
11	118.95	6.96 (1H, dd, J = 6.3~2.2 Hz)	23.64	1.92 (2H, m)
12	132.65	7.11(1H, dd, J = 6.3~2.2 Hz)	122.50	5.12 (1H, t, J = 3.5 Hz)
13			143.75	
14			40.08	
15			32.92	1.90 (2H, m)
16			38.52	1.97 (2H, d, J = 11.5 Hz)
17			36.84	
18			42.13	
19			41.57	1.89 (2H, m)
20			41.57	
21			139.67	5.18 (1H, t, J = 3.5 Hz)
22			124.37	5.34 (1H, t, J = 3.5 Hz)
23			23.26	1.25 (3H, s)
24			16.91	1.13 (3H, s)
25			16.76	1.06 (3H, s)
26			17.53	0.98 (3H, s)
27			17.60	0.97 (3H, s)
28			23.61	0.91 (3H, s)
29			21.41	0.87 (3H, s)
30			21.32	0.86 (3H, s)
CO-$\underline{C}H_3$			15.76	2.04 (3H, s)
$\underline{C}O$-CH_3			171.01	

Proton resonance integral, multiplicity, and coupling constant (J = Hz) are in parentheses.

present in preparations of *Calotropis gigantea* flower.

Similarly, compound **2** was obtained as white crystal. Its EI-MS showed a molecular ion (M^+) peak at *m/z* 466.4023. Its ^{1}H-NMR spectrum exhibited three olefinic protons at δ 5.34, 5.14 and 5.12 and an acetyl methyl protons at δ 2.04. The ^{13}C-NMR showed an acetyl carbonyl carbon at δ 171.01, four olefinic carbons at δ 122.5 (C-12), 143.75 (C-13), 139.67 (C-21) and 124.37 (C-22) and acetyl methyl carbon at 15.76 (Table 1). Based on the foregoing observations and a comparison of the data with the literature (Kim *et al.*, 2004), compound **2** was determined to be anhydrosophoradiol-3-acetate. Isolation of both DEHP and anhydrosophoradiol-3-acetate is reported for the first time from this plant.

In vitro antibacterial activity study, both ethyl acetate extract and compound **1** showed a better broad spectrum of antibacterial activity against both Gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, and *Sarcina lutea*) and Gram negative (*Escherchia coli*, *Shigella sonnei*, *Shigella shiga* and *Shigella dysenteriae*) bacteria, with inhibition zones in the range of 07~20 mm (Table 2). Although ethyl acetate extract showed activity, compound **1** was inactive against *Bacillus megaterium*. Compound **2** showed moderate activity against *Staphylococcus aureus*, *Sarcina lutea* and *Escherchia coli*. It produced inhibition zone ranging from 08 to 15 mm (Table 2). Minimum inhibitory concentration (MIC) values were also evaluated against four Gram positive and four Gram negative bacteria. The lowest MIC values were observed for ethyl acetate extract (16 μg/*ml*) and compound **1** (32 μg/*ml*)

Table 2. *In vitro* antibacterial activity of the extract and isolated compounds

Microorganisms	Zone of Inhibition									
	Ethyl acetate extract			Compound **1**			Compound **2**			Kanamycin
	Dose (μg/disc)									
	30	60	90	30	60	90	30	60	90	30
Staphylococcus aureus	08 ± 1.0	11 ± 0.5	15 ± 0.5	09 ± 0.3	12 ± 0.6	14 ± 0.5	10 ± 0.6	12 ± 0.3	15 ± 0.6	31 ± 1.0
Bacillus subtilis	10 ± 1.7	16 ± 0.8	18 ± 1.0	13 ± 0.6	16 ± 0.5	18 ± 0.6	R	R	R	27 ± 1.0
Bacillus megaterium	07 ± 0.6	09 ± 0.7	12 ± 1.1	R	R	R	R	R	R	26 ± 1.1
Sarcina lutea	12 ± 0.6	16 ± 0.6	19 ± 0.6	12 ± 0.6	16 ± 0.3	20 ± 0.5	08 ± 0.6	11 ± 0.5	13 ± 1.0	27 ± 0.6
Escherchia coli	13 ± 1.1	17 ± 0.6	20 ± 0.8	10 ± 0.3	13 ± 0.5	15 ± 0.6	09 ± 0.6	11 ± 0.7	14 ± 0.6	32 ± 1.1
Shigella sonnei	10 ± 0.6	14 ± 0.5	16 ± 0.6	11 ± 0.6	13 ± 1.0	17 ± 0.6	R	R	R	29 ± 0.6
Shigella shiga	08 ± 1.0	10 ± 0.8	20 ± 0.6	12 ± 1.1	15 ± 0.6	19 ± 0.3	R	R	R	31 ± 1.7
Shigella dysenteriae	09 ± 0.6	12 ± 0.3	14 ± 0.5	07 ± 0.6	10 ± 0.6	13 ± 1.0	R	R	R	28 ± 1.5

Data are expressed as mean ± S.E (Standard error); R = Resistance.

Table 3. Minimum inhibitory concentrations (MICs) of the extract and isolated compounds

Microorganisms	Ethyl acetate extract (μg/*ml*)	Compound **1** (μg/*ml*)	Compound **2** (μg/*ml*)
Staphylococcus aureus	32	64	64
Bacillus subtilis	16	32	–
Bacillus megaterium	128	–	–
Sarcina lutea	16	32	128
Escherchia coli	32	64	128
Shigella sonnei	32	64	–
Shigella Shiga	64	64	–
Shigella dysenteriae	128	128	–

Table 4. *In vitro* antifungal activity of the extract and isolated compounds

Microorganisms	Zone of inhibition									
	Ethyl acetate extract			Compound **1**			Compound **2**			Nystatin
	Dose (μg/disc)									
	100	200	400	100	200	400	100	200	400	100
Aspergillus flavus	10 ± 0.8	12 ± 0.3	15 ± 0.7	08 ± 0.3	11 ± 0.6	13 ± 0.6	R	R	R	26 ± 1.1
Aspergillus fumigatus	07 ± 0.6	09 ± 0.3	12 ± 0.3	R	R	R	R	R	R	30 ± 1.0
Aspergillus niger	R	R	R	R	R	R	R	R	R	25 ± 0.6
Fusarium sp.	R	R	R	R	R	R	R	R	R	31 ± 0.3

Data are expressed as mean ± S.E (Standard error); R = Resistance.

Table 5. Cytotoxicity of the extract and isolated compounds against brine shrimp nauplii

Sample	LC_{50} ($\mu g/ml$)
Amphicillin trihydrate	7.21 ± 0.47
Ethyl acetate extract	14.61 ± 0.71
Compound **1**	9.19 ± 0.42
Compound **2**	15.55 ± 0.37

Data are expressed as mean ± S.E (Standard error)

against *Bacillus subtilis* and *Sarcina lutea* (Table 3). Compound **2** showed lowest MIC value (64 $\mu g/ml$) against *Staphylococcus aureus* (Table 3). In antifungal activity test, ethyl acetate extract produced zone of inhibition between 07 to 15 mm against *Aspergillus flavus* and *Aspergillus fumigatus* whereas compound **1** exhibited activity against *Aspergillus flavus* (Table 4). Compound **2** had no antifungal activity.

Ethyl acetate extract, compound **1** and compound **2** showed toxicity against brine shrimp nauplii (*Artemia salina*). Among the samples, compound **1** showed the highest toxicity and LC_{50} value was 9.19 ($\mu g/ml$). Ethyl acetate extract and compound **2** exhibited moderate activity in comparison with ampicillin trihydrate (Table 5).

In previous study, Sastry and Rao (Sastry and Rao, 1995) showed the activity of DEHP (compound **1**) against *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella typhimurium* and *Pseudomonas aurioginosa*. The present study revealed the antishigellosis activity of DEHP because it had better activity against *Shigella shiga*, *Shigella sonnei* and *Shigella dysenteriae*. The DEHP is considered as pro inflammatory agent in other studies (Gourlay *et al.*, 2003; Oie *et al.*, 1997). Researchers also showed that anhydrosophoradiol-3-acetate (compound **2**) exhibited potent cytotoxicity against A549, SK-OV-3, SK-MEL-2, MES-SA and HCT-15 tumour cell lines (Kim *et al.*, 2004). So the overall findings of this study make important contribution in proper use of *Calotropis gigantea* flower for better health care system of common people in Bangladesh.

References

Adak, M., and Gupta, J. K. 2006. Evaluation of anti-inflammatory activity of *Calotropis gigantea* (AKANDA) in various biological systems. *Nepal. Med. Coll. J.* 8:156-61.

Anjaneyulu, V. and Row, L. R. 1968. The triterpenes of *Calotropis gigantea* Linn. *Curr. Sci.* 6:156-157.

Amade, P., Mallea, M. and Bouaicha, N. 1994. Isolation, structure identification and biological activity of two metabolites produced by *Penicillum olsonii* Bainier and Sartory. *J. Antibiot.* 47:201-207.

Argal, A. and Pathak, A. K. 2006. CNS activity of *Calotropis gigantea* roots. *J. Ethnopharmacol.* 106:142-145.

Basile, A., Giordano, S. Lopez-Saez, J. A. and Cobianchi, R. C. 1999. Antibacterial activity of pure flavonoids isolated from mosses. *Phytochemistry* 52:1479-1482.

Basu, K. P. and Nath, M. C. 1934. Calosterol, a sterol present in the milky juice of *Calotropis gigantea*. *Biochem. J.* 28:1561-1564.

Chitme, H.R., Chandra, R. and Kaushik, S. 2005. Evaluation of antipyretic activity of *Calotropis gigantea* (Asclepiadaceae) in experimental animals. *Phytother. Res.* 19:454-456.

Chitme, H. R., Chandra, R. and Kaushik, S. 2004. Studies on anti-diarrhoeal activity of *Calotropis gigantea* R.Br. in experimental animals. *J. Pharm. Pharm. Sci.* 7:70-75.

Ghani, A., 2003. Medicinal Plants of Bangladesh, pp. 141. Asiatic Society of Bangladesh, Dhaka.

Gourlay, T., Samartzis, I., Stefanou, D. and Taylor, K. 2003. Inflammatory response of rat and human neutrophils exposed to di-(2-ethyl-hexyl)-phthalate-plasticized polyvinyl chloride. *Artificial Organs* 27:256-260.

Gupta, J. and Ali, M. 2000. Rare chemical constituents from *Calotropis gigantea* roots. *Indian. J. Pharm. Sci.* 62:29-32.

Habib, M. R., Nikkon, F., Rahman, M., Haque, M.E. and Karim, M.R. 2007. Isolation of stigmasterol and β-sitosterol from methanolic extract of root bark of *Calotropis gigantea* (linn). *Pakistan J. Biol. Sci.* 10:4174-4176.

Karaman, I., Sahin, F., Gulluce, M., Ogutcu, H., Sngul, M. and Adiguzel, A. 2003. Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *J. Ethnopharmacol.* 85:231-235.

Kartikar, K. R. and Basu, N. 1994. Indian Medicinal Plants, pp. 1607 Lolit Mohan Basu, Allahabad, India.

Kim, M., Lee, H., Hahm, K., Moon, Y. and Woo, E. 2004. Pentacyclic triterpenoids and their cytotoxicity from the stem bark of *Styrax japonicas*. et Z. *Arch. Pharm. Res.* 27:283-286.

Kitagawa, I., Zhang, R. S., Park, J. D., Baek, N. I., Takeda, Y., Yoshikawa, M. and Shibuya, H. 1992. Indonesian medicinal plants. I. Chemical structures of calotroposides A and B, two new oxypregnane-oligoglycosides A and B, from the root of *Calotropis gigantea* (Asclepiadaceae). *Chem. Pharm. Bull.* (Tokyo) 40:2007-2013.

Kiuchi, F., Fukao, Y., Maruyama, T., Obata, T., Tanaka, M., Sasaki, T., Mikage, M., Haque, M. E. and Tsuda, Y., 1998. Cytotoxic principles of a Bangladeshi crude drug, akond mul (roots of *Calotropis gigantea* L.). *Chem. Pharm. Bull.* (Tokyo) 46:528-530.

Lee, K. H., Kim, J. H., Lim, D. S. and Kim, C. H. 2000. Anti-leukaemic and anti-mutagenic effects of Di-(2-ethylhexyl) phthalate isolated from *Aloe vera* Linn. *J. Pharm. Pharmacol.* 52:593-598.

Lhinhatrakool, T. and Sutthivaiyakit, S. 2006. 19-Nor- and 18, 20-epoxy-cardenolides from the leaves of *Calotropis gigantea*. *J. Nat. Prod.* 69:1249-51.

Mavar-Manga, H., Haddad, M., Pieters, L., Baccelli, C., Penge, A. and Quetin-Lectercq, J. 2008. Anti-inflammatory compounds from leaves and root of *Alchornea cordifolia* (Schumach. & Thonn.) Müll. *Arg. J. Ethnopharmacol.* 115:25-29.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E. and Mclaughlin J. L. 1982. Brine shrimp: a convenient general bioassay for active plant constituents, *Planta. Med.* 45:31-33.

Mueen, A. K. K., Rana, A. C. and Kixit, V. K. 2005. Calotropis species (Asclepiadaceae)-A comprehensive review. *Pharmacognosy Magazine* 1:48-52.

Mukherjee, P. K., Saritha, G. S. and Suresh, B. 2002. Antimicrobial potential of two different *Hypericum* species available in India. *Phytother. Res.* 16:692-695.

Oie, L., Hersoug, L. G. and Madsen, J. O. 1997. Residential exposure to plasticizers and its possible role in the pathogenesis of asthma. *Environ. Health Perspect.* 105:972-978.

Pari, K., Rao, P. J., Devakumar, C. and Rastogi, J. N. 1998. A novel insect antifeedant nonprotein amino acid from *Calotropis gigantea*. *J. Nat. Prod.* 61:102-104.

Pathak, A. K. and Argal, A. 2007. Analgesic activity of *Calotropis gigantea* flower. *Fitoterapia* 78:40-42.

Rao, G. N., Kumar, P. M., Dhandapani, V. S., Krishna, T. R. and Hayashi, T. 2000. Constituents of *Cassia auriculata*. *Fitoterapia* 71:82-83.

Reiner, R. 1982. Detection of antibiotic activity. In: Antibiotic - An Introduction, pp. 21-27. Roche Scientific Services, Switzerland.

Rois, J. J., Reico, M. C. and Villar A. 1988. Antimicrobial Screening of natural products, *J. Enthopharmocol.* 23:127-149.

Sastry, V. M. V. S. and Rao, G. R. K. 1995. Dioctyl phthalate and antibacterial compound from the marine brown alga-*Sargassum wightii*. *J. Appl. Phycol.* 7:185-186.

Sen, S., Sahu, N. P. and Mahato, S. B. 1992. Flavonol glycosides from *Calotropis gigantea*. *Phytochemistry* 31:2919-2921.

Shibuya, H., Zhang, R., Park, J. D., Back, N. I., Takeda, Y., Yoshikawa, M. and Kitagawa, I. 1992. Chemical structures of Calotroposides C, D, E, F and G, five additional new oxypregnane-oligoglycosides from the root of *Calotropis gigantea* (Asclepiadaceae). *Chem. Pharm. Bull.* 40:2647-2653.

Thakur, S., Das, P., Itoh, T., Imai, K. and Matsumoto, T. 1984. Latex extractables of *Calotropis gigantea*. *Phytochemistry* 23:2085-2087.

Toth-Soma, L. T., Gulyas, S., Szegletes, Z. 1993. Functional connection between and extracellular secretion in species of *Euphorbia* genus. *Acta. Biol. Hung.* 44:433-443.