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Studies on cytotoxic, phytotoxic and volatile profile of the bark extract of the medicinal plant, *Mallotus tetracoccus* (Roxb.) Kurz.

Subbiah Ramalakshmi and Krishnaswamy Muthuchelian*

Department of Bioenergy, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai - 625 021, India.

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This study was aimed at analysing the compounds present in the bark extract of *Mallotus tetracoccus* (Roxb.) Kurz. by GC-MS analysis and also to investigate the cytotoxic and phytotoxic activity of *Mallotus tetracoccus* (Roxb.) Kurz. bark extract. The major constituents in *M. tetracoccus* (Roxb.) Kurz. bark extract are thiocyanic acid and 2-propynyl ester (52.04%). It possesses biocidal, antioxidative, antimutagenic and anticancer activity. The cytotoxic activity of bark extract was evaluated by brine shrimp lethality bioassay method and the LC_{50} value was found to be 84.72 µg/ml compared to taxol 0.85 µg/ml. Phytotoxicity assay showed significant root length inhibition by the extract at the concentrations of 100, 1000 and 10000 ppm. Similarly, seed germination studies shows that the bark extract possess significant inhibition at concentrations of 1000 and 7500 ppm.

Key words: *Mallotus tetracoccus*, GC-MS analysis, thiocyanic acid, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl, cytotoxicity, phytotoxicity, radish seed, artemia salina.

INTRODUCTION

Interactions between higher plants take place either by competition or by chemical inhibition (Mancini et al., 2009). When the effect is due to the release of an effective phytotoxin, it is called allelopathy. Small quantities of toxins are responsible for massive reductions in plant growth. Plants generally have inhibitory effects on neighbouring plants by releasing allelopathic chemicals into the soil (Harborne, 1988; Inderjit, 1996; Seigler, 1996). Allelochemicals inhibit germination and seedling growth probably by affecting cell division and elongation, processes that are very important at this stage, or by interfering with enzymes involved in the mobilization of nutrients necessary for germination (Batlang and Shushu, 2007). Thus, the phytotoxicity of the bark extract of *Mallotus tetracoccus* was studied using radish seed for root length and seed germination determination.

M. tetracoccus (Roxb.) Kurz. is found in Western Ghats of India. *M. tetracoccus* is one of the medicinally important plants belonging to the family Euphorbiaceae, commonly known as “vatta kanni” in Tamil. Several species of the genus *Mallotus* are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans and chalcones (Amakura and Toshida, 1996; Tanaka et al., 1998; Huang et al., 1999; Cheng and Chen, 1999; Wei et al., 2004; Ma et al., 2004; Likhitwitayawuid and Supudompol, 2005). *M. tetracoccus* (Roxb.) Kurz, are found in evergreen forests up to 1600 m. The common names include *Mullu polavu,* Vatta (Tamil), *Thavatta,*

*Corresponding author. E-mail: drchelian1960@yahoo.co.in. Tel: +91 452 2458020.

Vatta, Vatta kumbil, Vetta kumbil (Malayalam) and *Uppale mara* (Kannada). The trees grow up to 5 to15 m tall, leaf blades are triangular-ovate or ovate, sometimes 1- or 2-lobate, 10-25 × 9 to 20 cm, leathery, abaxially brownish tomentose, adaxially glabrous, base obtuse or truncate. The reported bioactivities of the extracts or the individual chemical constituents isolated from this genus include antipyretic (Chattopadhyay et al., 2002), anti-inflammatory, hepatoprotective (Kim et al., 2000), antioxidant and radical scavenging activities (Arfan et al., 2007).

The active compounds present in the *M. tetracoccus* ethanolic leaf extract showed the presence of various chemical constituents such as Bis (2-ethyl hexyl) phthalate (46.78%), 3-methyl-2-(2-oxypropyl) furan (13.31%), E-8-methyl-9-tetradecen-1-ol acetate (6.63%), Octadecanoic acid, 2-oxo (4.46%) and Longiborneol (2.39%) (Ramalakshmi and Muthuchelian, 2011b).

The study report of the *M. tetracoccus* bark (MTB) extract in our laboratory showed to have significant antioxidant, antimicrobial and radical scavenging activities (Ramalakshmi and Muthuchelian, 2012). Thus, the objective was to analyse the cytotoxicity, phytotoxicity and volatile profile of the MTB extract.

MATERIALS AND METHODS

Collection of plant material

The fresh bark of *M. tetracoccus* (Roxb.) Kurz. were collected from the Agasthiar Malai reserved forest, Western Ghats, South India, authenticated by Prof. Dr. K. Muthuchelian, Director, Centre for Biodiversity and Forest Studies, Madurai Kamaraj University, and voucher specimens were deposited in the herbarium of Centre for Biodiversity and Forest Studies of our university (No.AM-07).

Preparation of extract

Fresh barks were shade dried, powdered and extracted with ethanol for 6 to 8 h using soxhlet apparatus. The extract was then filtered through muslin, evaporated under reduced pressure and vacuum dried to get the viscous residue. The ethanolic extract of the bark was used for cytotoxic, phytotoxic studies and GC-MS analysis.

GC-MS analysis

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1), injector temperature was 250°C and ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10 to 200°C/min, then 5 to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62 000 patterns.

Cytotoxicity bioassay

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Twenty nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted with a hand lens. Experiments were conducted along with control (vehicle treated), different concentrations (10 to 1000 µg/ml) of the test substances in a set of three tubes per dose. Based on the percent mortality, the LD_{50} of the test compound was determined using probit scale (Wardlaw, 1985).

Radish seed phytotoxicity assay

The phytotoxic properties of MTB extract was evaluated using radish seed phytotoxicity assay (Turker and Camper, 2002; Islam et al., 2009). Two type of determination were done for this purpose:

Root length determination

Radish seed was washed with distilled water and with 1% mercuric chloride. Whatman No. 1 filter paper kept on Petri dish and 5 ml extracts (100, 1000 and 10000 ppm) were added separately. Filter paper was dried at room temperature for reducing extra solvent. 5 ml double distilled water was added and then 20 radish seeds were placed on Petri dishes followed by tight sealing and incubation at 23 ± 2°C. Root length was measured after 1, 3 and 5 days of interval. Only double distilled water containing Petri dish was used as control. Each assay was carried out in three times.

Seed germination determination

This part of the determination is similar to that of earlier determination except for the extract concentrations and number of seeds. Here, two different concentrations (1000 and 7500 ppm) and 100 radish seeds were used. Germinated seeds were counted after every day up to 5 days. Each experiment was carried out three times.

Statistical analysis

Results were expressed as the means of three replicates ± the standard deviation of triplicate analysis.

RESULTS AND DISCUSSION

GC-MS analysis

On comparison of the mass spectra of the constituents with the NIST library, five peaks were obtained; all the phytoconstituents were characterized and identified

Table 1. Phytocomponents identified in the ethanolic bark extract of Mallotus tetracoccus (Roxb.) Kurz. by GC-MS.

Number	RT	Name of the compound	Peak area (%)
1	12.45	Furfural	28.31
2	19.48	4H- Pyran-4 -one, 2,3-dihydro-3,5-dihydroxy-6-methyl	8.70
3	21.02	Thiocyanic acid, 2-propynyl ester	52.04
4	24.15	Benzofuran, 7(2,4-dinitrophenoxy)- 3- ethoxy- 2,3- dihydro-2-dimethyl	6.38
5	24.52	Benzaldehyde, 3-hydroxy-4-methoxy	4.57

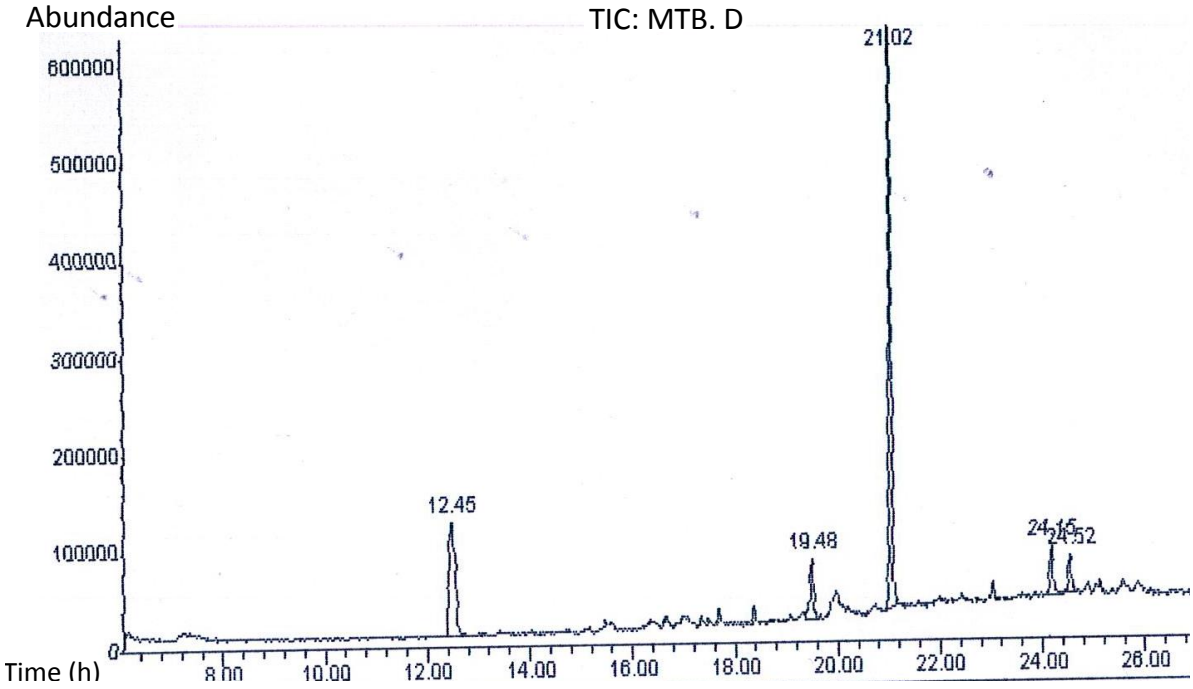

Figure 1. GC-MS Chromatogram of the ethanolic bark extracts of *M. tetracoccus* (Roxb.) Kurz.

(Table 1). GC-MS chromatogram of the MTB is given in Figure 1. The retention times (RT) are in minutes. The major chemical constituents in ethanolic bark extract studied through GC-MS are thiocyanic acid-2-propynyl ester (52.04%), furfural (28.31%), 4H-pyran-4 -one-2,3-dihydro-3,5-dihydroxy-6-methyl (8.70%), benzofuran-7-(2,4-dinitrophenoxy)-3-ethoxy-2,3-dihydro-2-dimethyl (6.38%) and benzaldehyde-3-hydroxy-4-methoxy (4.57%).

The major constituents, thiocyanic acid, and 2-propynyl ester were found at retention time of 21.02 min. Glucosinolates are organic anionic compounds containing sulphur, nitrogen and a group derived from glucose (Kjaer, 1960; Ettlinger and Kjaer, 1968). Glucosinolates are found in all parts of the plant (Kjaer, 1976) and up to 15 different types of glucosinolates have been found in the same plant. Glucosinolates and myrosinase enzyme come in contact when plant tissue is damaged leading to formation of hydrolytic products of glucosinolates (Kaur et al., 2011). The breakdown products of glucosinolates when exposed to myrosinase enzyme include isothiocyanates, nitriles, epithionitriles, and thiocyanates, which are known to possess wide array of biological activities such as biocidal (Vig et al., 2009), antioxidative (Barillari et al., 2005), antimutagenic (Rampal et al., 2010) and anticancer activities (Rosea et al., 2005).

The second main active constituent, furfural (28.31%) was found at retention time of 12.46 min. The antifungal activities of furfural and its derivative have been reported discussing their feasibilities for antifungal treatment (Jouad et al., 2001; Moon et al., 1993). The pine needle extract contained four chemical compounds of which furfural are the main constituent. The extracts were reported to possess significant antifungal activity against plant pathogen fungus, *Alternaria mali* (Jung et al., 2007). The 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6-methyl, a flavonoid compound found at retention time of 19.47 min is said to possess antimicrobial and anti-inflammatory activities (Praveen Kumar et al., 2010; Ramalakshmi and Muthuchelian, 2011a). Benzofuran,

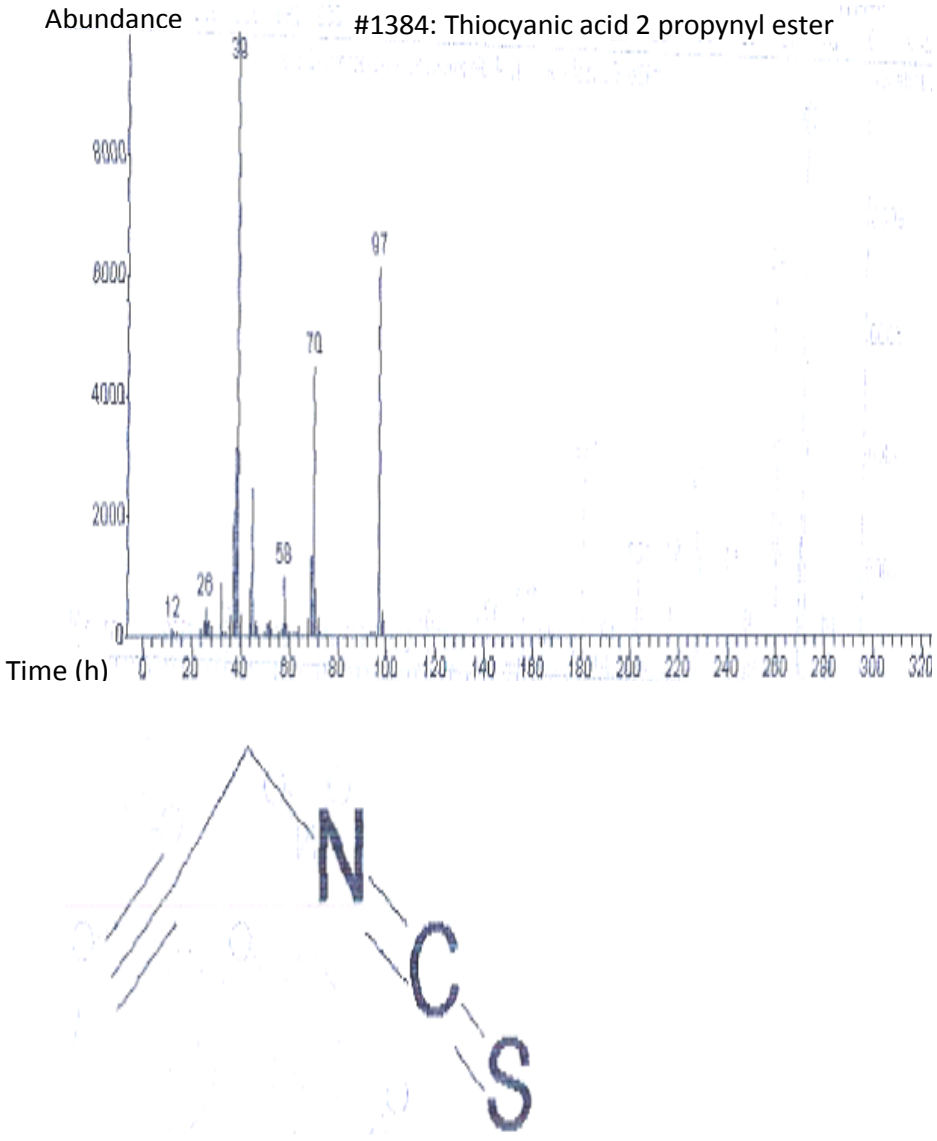

Figure 2. The mass spectrum analysis and structure of thiocyanic acid, 2-propynyl ester.

7(2, 4-dinitrophenoxy)- 3- ethoxy- 2, 3- dihydro-2-dimethyl, a coumaran, is said to possess activities such as antihelminthic, anti-inflammatory, and anti-diarrhoeal activities (Ramalakshmi and Muthuchelian, 2011a). The major phytochemical constituents, thiocyanic acid, 2-propynyl ester, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl present in ethanolic extract of MTB is presented as mass spectra and compound structures in Figures 2, 3 and 4.

Cytotoxicity bioassay

A general bioassay capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLT) (Hamid et al., 2011). The cytotoxicity bioassay against *Artemia salina* is a simple and inexpensive method to test cytotoxicity, to biodirect fractionation of natural products and as a predictor of antitumor and pesticidal activity (Sanchez et al., 1993). The ethanolic MTB extract shows significant cytotoxic activity against brine shrimp and the LC_{50} value was found to be 84.72 µg/ml compared to taxol 0.85 µg/ml (Figure 5).

The inhibitory effect of the MTB extract might be due to the presence of toxic compounds such as thiocyanic acid, 2-propynyl ester, furfural and 4H- pyran-4 -one, 2, 3-dihydro-3, 5- dihydroxy-6- methyl present in the extract possessing antitumor, antimicrobial, antioxidant and anti-inflammatory activity. So the cytotoxic effects of the bark extract enunciate that it can be selected for further cell line assay because there is a correlation between cytoto-

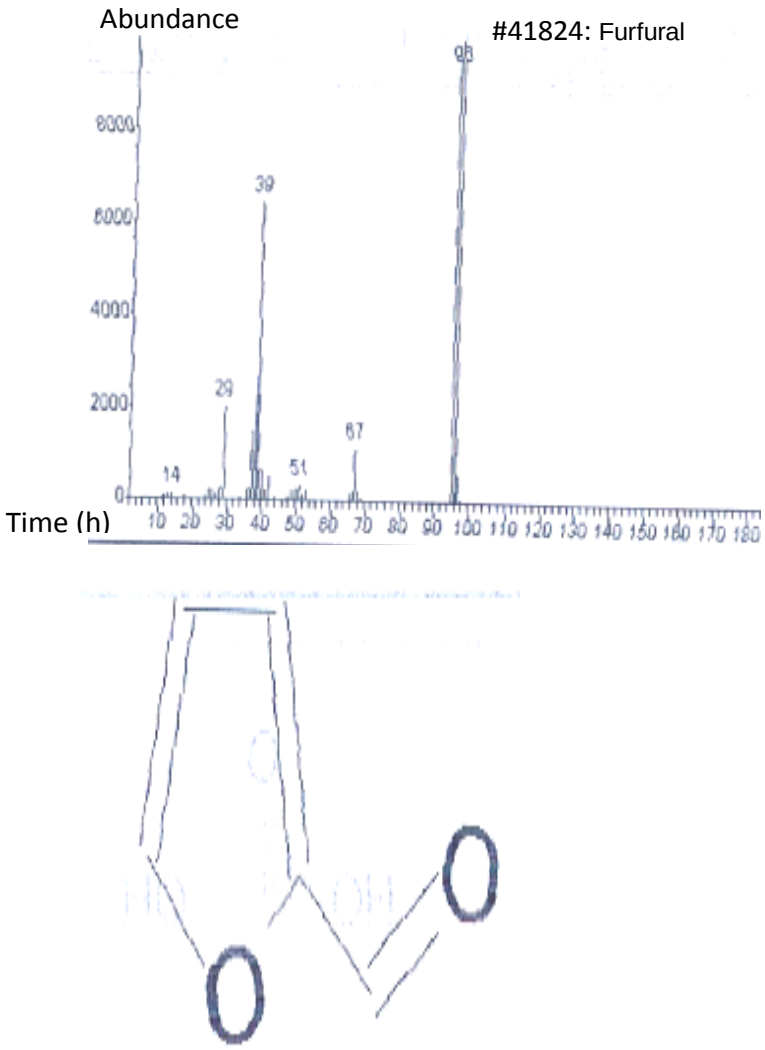

Figure 3. The mass spectrum analysis and structure of furfural.

Xicity and activity against the brine shrimp nauplii using extracts (Manilal et al., 2009; Haque et al., 2009). The results on brine shrimps assay indicate that the extract has LC_{50} value greater than 20 µg/ml; the recommended cut-off point for detecting cytotoxic activity (Geran et al., 1972).

On comparison of our study results with other research work, our extract possessed significant cytotoxic activity. The cytotoxic potential (ED_{50}) of different fractions [crude methanolic extract (CME), n-Hexane fraction (NHF) and aqueous fraction (AQF)] of *Aster thomsonii*, the AQF values were found to possess maximum activity of 154.69 µg/ml (Bibi et al., 2011). Several other cytotoxicity studies show that the results of *Thymus serpyllum*, 466 µg/ml (Rehman et al., 2009), and out of 60 medicinal plants from brazil screened for activity showed that only 10% plants showed ED_{50} < 1000 µg/ml (Maria et al., 2000). Brine shrimp lethality bioassay of petroleum ether and methanol extracts of the seeds of *Khaya senegalensis* possessed significant cytotoxicity LC_{50} values of 827.39 and 51.79 µg/ml, respectively (Juss et al., 2007). The LC_{50} values of standard Vincristin sulphate, petroleum ether, chloroform and ethyl acetate extracts of *Marsilea quadrifolia* were 6.628, 9.543, 7.820 and 8.589 µg/ml respectively (Ripa et al., 2009). The cytotoxic potential of aqueous extract of *Ficus racemosa* seed showed an LC_{50} value of 4.04 µg/ml (Hamid et al., 2011).

Radish seed phytotoxicity assay

Phytotoxicity is an important attribute in determination of allelopathic potential of a plant species (Khan et al., 2011). It is a common tradition that easily grown, sensitive, reliable species like *Lemna minor*, Lettuce (*Lactuca sativa*) and radish (*Raphanus sativa*) seeds are used as test plants in allelopathic studies (Putnam et al., 1983; Einhelling et al., 1985; Leather and Einhelling, 1985).

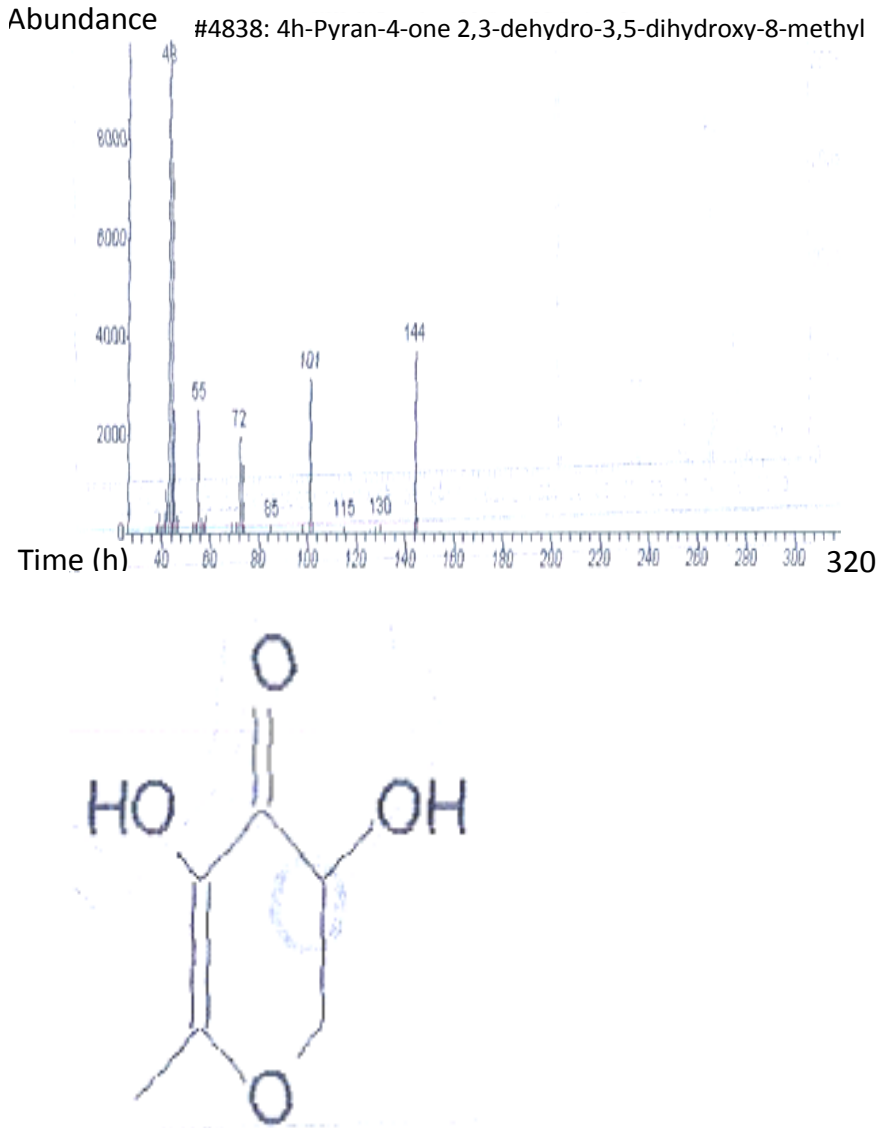

Figure 4. The mass spectrum analysis and structure of 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl.

This radish seed phytotoxicity assay has a wide range of application in research towards the discovery of active principles in plants (Arzu et al., 2002). The root lengths of radish seeds germinated were significantly inhibited by the bark extracts at concentrations of 100, 1000 and 10000 ppm (Figure 6). Similarly, the seed germination inhibition was said to be significantly high when compared to control (Figure 7). The MTB extract exhibited significant phytotoxicity on radish seeds due to the presence of phytochemicals such as thiocyanic acid, furfural and 4H- pyran-4 -one, 2, 3- dihydro-3, 5- dihydroxy-6- methyl. Similarly, the allyl isothiocyanates (ITC) isolated from black mustard (*Brassica nigra* L.) residues inhibited establishment of grass species. Benzyl-ITC, a break down product of white mustard (Josefsson, 1968; Tollsten, 1988) was phytotoxic to velvet leaf, sicklepod (*Senna obtusifolia*) and sorghum. Other break down products of glucosinate like ionic thiocyanate (SCN-) inhibited the root or shoot growth of many crop species (Brown et al., 1991).

Aqueous extracts of *Nicotiana glauca* Graham (stems, roots and fruits) was evaluated for phytotoxicity on two crops (lettuce and radish), where percentage inhibition was between 15 and 100%, due to the presence of phenolics (Rinez et al., 2011). Root length inhibition was more obvious than shoot length, as root length is a more sensitive indicator of phytotoxic activity (Rinez et al., 2011). The study by Turk et al. (2005) investigated the allelopathic effects of various black mustard (*Brassica nigra* L.) plant parts (leaf, stem, flower and root), where

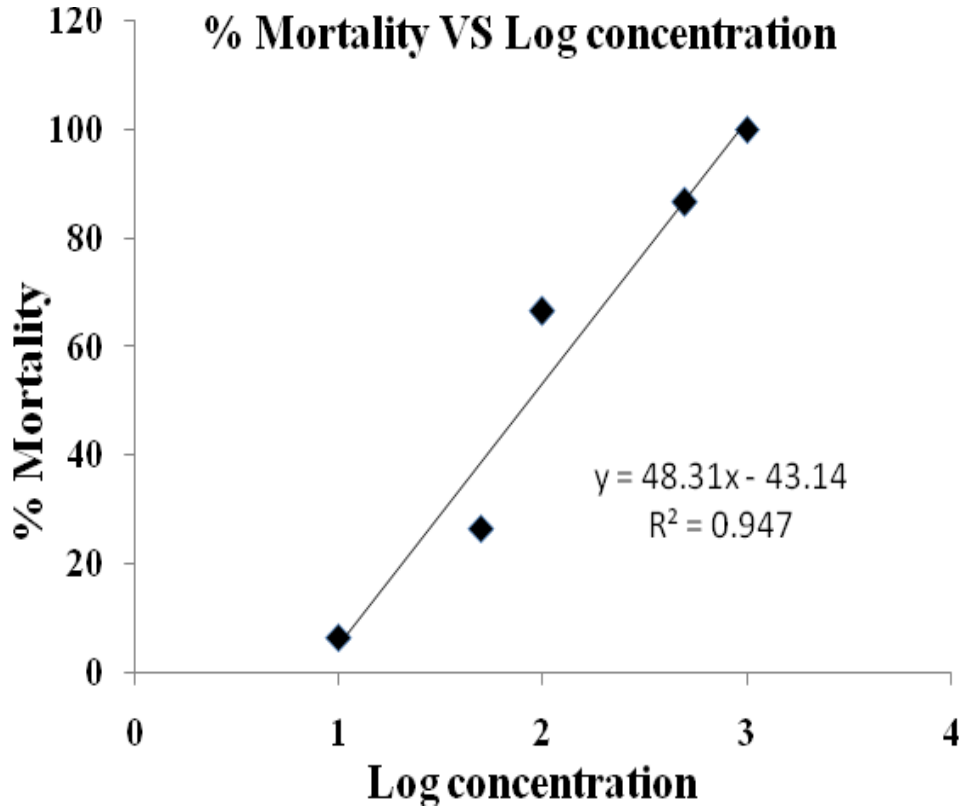

Figure 5. The toxicity effects of the *M. tetracoccus* (Roxb.) Kurz. bark extract using brine shrimp lethality assay after 24 h.

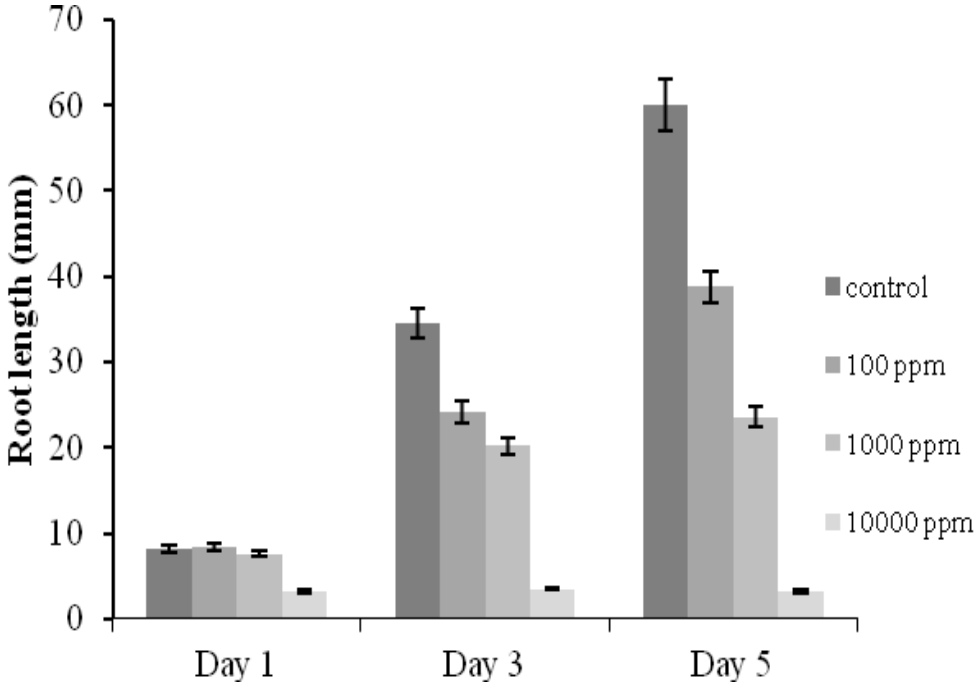

Figure 6. Histogram shows regular root length inhibition by the bark ethanol extract of *M. tetracoccus* at different concentrations (100, 1000 and 10000 ppm). Data was compared with the control.

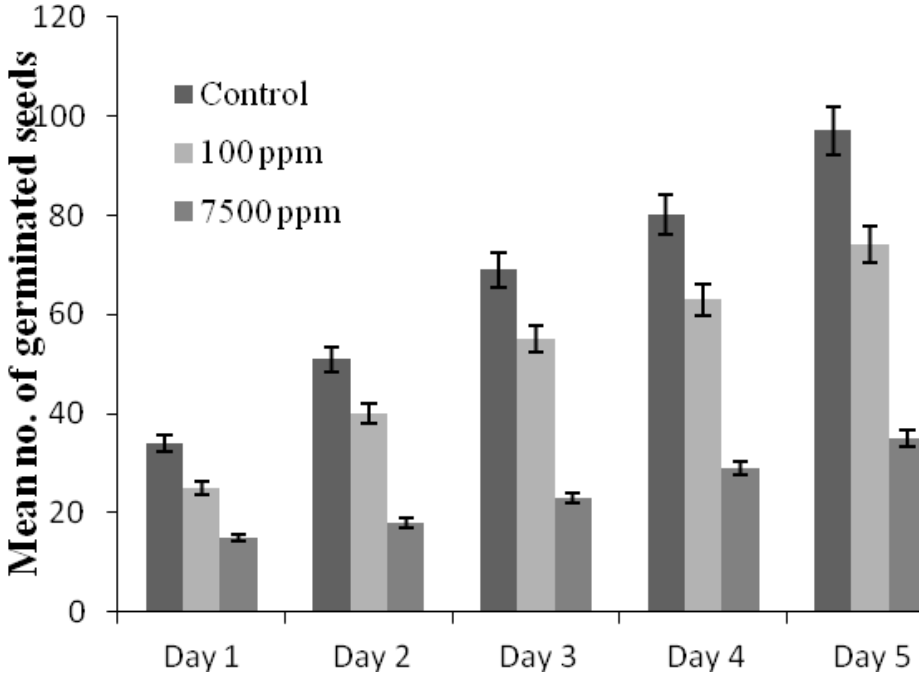

Figure 7. Graph showing phytotoxicity assay on radish seed germination percentage at two different concentrations (1000 and 7500 ppm) of ethanolic bark extract of Mallotus tetracoccus. Data was compared with the control.

the aqueous extracts significantly inhibited radish seed germination and seedling growth when compared with distilled water control. The aqueous root extract of *Ailanthus altissima* was purified to give active compounds such as ailanthone, ailanthinone, chaparrine, and ailanthinol B (quassinoid derivatives), where the alkaloid 1-methoxycanthin-6-one is not active. Then, the compounds where studied for the allelopathic activity using radish, garden cress and purslane seeds, where ailanthone showed greatest inhibitory activity (Feo et al., 2003). Feo et al. (2003) through his studies has reported that out of three seeds studied for phytotoxicity, radish seeds was the most sensitive to allelochemicals.

The essential oils of *S. hierosolymitana* Boiss. and *S. multicaulis* Vahl. var. simplicifolia Boiss. was studied for the phytotoxic effects on *Raphanus sativus* L. (radish) and *Lepidium sativum* L. (garden cress), where the extract inhibited and promoted radish seed germination at doses of 0.625 and 0.24 µg/ml, respectively (Mancini et al., 2009).

Conclusion

GC-MS analysis was found useful in the identification of several constituents such as thiocyanic acid, 2-propynyl ester (52.04%), furfural (28.31%), 4H- Pyran-4 -one, 2, 3-Dihydro-3, 5- dihydroxy-6- methyl (8.70%), benzo-furan, 7 (2, 4-dinitrophenoxy)- 3- ethoxy- 2, 3- dihydro-2- dimethyl (6.38%) and benzaldehyde, 3-hydroxy-4-methoxy (4.57%) present in the ethanolic extract of MTB. The cytotoxic activity of ethanolic extract of MTB was assessed by using brine shrimp, *Artemia salina*, where the bark (84.72 µg/ml) was said to possess significant activity compared to taxol (0.85 µg/ml). The presence of major bioactive compound, thiocyanic acid and furfural justifies the use of the whole plant for various ailments by traditional practitioners. The phytotoxic activity of *MTB* is probably due to the presence of a substantial amount of thiocyanic acid, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl, benzofuran and benzaldehyde. The result obtained from the brine shrimp lethality bioassay of MTB can be used as a guide for the isolation of cytotoxic compounds from the aqueous extract of the bark of this plant.

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