Toxic Potentiality of Bio-oils, from Biomass Pyrolysis, in Cultured Cells and *Caenorhabditis elegans*

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ABSTRACT: Bio-oils, which are multicomponent mixtures, were produced from two different biomass (rice straw (rice oil) and sawdust of oak tree (oak oil)) by using the slow pyrolysis process, and chemical compositional screening with GC-MS detected several hazardous compounds in both bio-oil samples. The two bio-oils vary in their chemical compositional nature and concentrations. To know the actual hazard potentialities of these bio-oils, toxicological assessments were carried out in a comparative approach by using *in vitro* (Jurkat T and HepG2 cell) as well as *in vivo* (*Caenorhabditis elegans*) systems. A dose-dependent increase in cytotoxicity, cell death (apoptosis), and genotoxicity were observed in cultured cell systems. Similarly, the *in vivo* system, *C. elegans* also displayed a dose-dependent decrease in survival. It was found that in comparison with rice oil, oak oil displayed higher toxicity to all models systems, and the susceptibility order of the model systems were Jurkat T $>$ HepG2 $>$ *C. elegans*. Pursuing the study further toward the underlying mechanism by exploiting the *C. elegans* mutants screening assay, the bio-oils seem to mediate toxicity through oxidative stress and impairment of immunity. Taken together, bio-oils compositions mainly depend on the feedstock used and the pyrolysis conditions which in turn modulate their toxic potentiality. © 2013 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2013.

Keywords: bio-oil toxicity; HepG2 cells; Jurkat T cells; *Caenorhabditis elegans*; mutant screening

INTRODUCTION

The increased demand of alternative energy sources has created interest in renewable energy sources such as biomass. Among the variety of methods used to convert biomass into useful energy products, pyrolysis of lignocellulosic biomass is one of the most recent and cheapest methods (Vispute et al., 2010). Pyrolysis is a thermal decomposition process that occurs at moderate temperatures with a high heat transfer rate in the biomass particles and produces a liquid product, bio-oil (Paul de Wild et al., 2011). Bio-oils, also known as pyrolytic oils or bio crudes, are usually dark brown, free-flowing liquids having a distinctive smoky odor (Dobele, 2002). These are multicomponent mixtures composed of different sized molecules and have actual elemental resemblance with the source biomass (Czernik and Bridgwater, 2004). The variety and range of products in bio-oil largely depend on the process temperature, pressure, and residence time of the vapors liberated during pyrolysis. By modifying pyrolysis conditions, storable, transportable, and comparatively eco-friendly (CO_2 neutral and low content of sulfur and nitrogen) bio-fuels can be produced. By performing further upgradation processes, such as fractionation, fine chemicals, petrochemicals, and automotive fuels can be produced

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(Czernik and Bridgwater, 2004; Huber et al., 2006). The upgraded bio-oil has already been successfully tested as a fuel for diesel engines, gas turbines, and boilers (Czernik and Bridgwater, 2004). In addition to high-quality fuel-oil, a number of high value compounds, phenols, organic acids, furfural, and levoglucosan can also be produced (Branca et al., 2011; Gary Brodeur, 2011). These byproducts have been used in various commercial purposes, for instance, nitrogenous fertilizer or soil conditioner, fungicide, bio-lime, etc. (Radlein and Majerski, 1997; Booker et al., 2010; Vitasari et al., 2012). One such byproduct is glycol-aldehyde, which has been extensively used in the food-flavoring industry as the most active meat-browning agent (Lu, 2010). The other promising byproduct, which can be produced up to 50% of total products by acid pre-treatment of high cellulose biomass, is levoglucosan (Dobele, 2002; Lu, 2010), which has potential in the synthesis of antibiotics and flavor compounds (Czernik and Bridgwater, 2004). Despite the use of bio-oil as fuel and as a number of associated commercial byproducts, however, poor volatility, high viscosity, coking, and corrosiveness are probably the most challenging limitations of bio-oil applications. Specifically, it is of utmost importance to understand the toxicological effects of bio-oils and their byproducts prior to their authorization and eventual large-scale usage (Cordella et al., 2012), which is also unavoidable in the frame of the European REACH system (Regulation (EC) No 1907/2006).

It is well accepted that the toxic potentiality of a particular compound depends on the model system used to evaluate the toxicity. Reliable, sensitive, and specific test systems therefore are needed, particularly for risk assessment. Jurkat T cell, a human lymphoma cell line, and HepG2 cell, a human hepatoma cell line, are frequently used in the toxicological assessment of environmentally relevant compounds ranging from organic pollutants to metallic compounds and nanomaterials (Song and Freedman, 2005; McDermott et al., 2008; Kawata et al., 2009; Liu and Zeng, 2009; Eom and Choi, 2010; Nemmiche et al., 2011; Bandele et al., 2012; Katika et al., 2012; Sharma et al., 2012). In the same context, *Caenorhabditis elegans (C. elegans)* is an emerging model in environmental toxicology, both for mechanistic studies as well as high-throughput screening. Various special features, such as well-characterized genome, genetic manipulability, ease of maintenance, short and prolific lifecycle, transparent and small body size, etc., have led to an increasing use of *C. elegans* in toxicology (Leung et al., 2008). Nowadays various research groups, including us, have successfully applied *C. elegans* as a toxicological model system for a diverse kind of environmentally relevant compounds (Roh et al., 2006, 2007; Cui et al., 2007; Pei et al., 2008; Wang et al., 2009; Boyd et al., 2010; Yang et al., 2011). In addition, a rich collection of available mutants made it possible to mutant screening assay, which are not only sensitive and useful in estimating the effects of toxicants but may also shed light on the underlying mechanisms (Leung et al., 2008; Roh et al., 2009).

At this level, only a limited number of studies are available about the hazardous effects of bio-oils obtained from biomass pyrolysis (Pimenta et al., 2000; Park et al., 2008; Bernardo et al., 2009). However, quite a moderate number of stuides were conducted with bio-disel and their specific blends on ecotoxicological model systems (reviewed by Khan et al., 2007).

In this study, we used two different bio-oils, one is rice oil pyrolysed from rice straw and another is oak oil produced from slow pyrolysis of sawdust of oak tree biomass. Next, the assessment of detailed toxicological potentialities, cytotoxicity, genotoxicity, and comparative toxicity, of two bio-oils were carried out in this study by using Jurkat T cells and HepG2 cells as *in vitro* systems and *C. elegans* as *in vivo* system. Moreover, mutant screening tests were performed by using a vast array of available *C. elegans* mutant strains.

MATERIALS AND METHODS

Bio-oils Preparation and Characterization

Rice straw and sawdust of oak tree were used as raw materials for the production of bio-oils. Prior to the pyrolysis, the raw materials were first roughly chopped into small pieces and then ground and sieved to obtain particles with diameters between 0.425 and 1 mm. The pyrolysis reactor was a fixed reactor made of a 316 SS tube with an inner diameter of 110 mm and a height of 390 mm and was equipped with a cyclone, a series of condenser and a gas-supplying system. In each pyrolysis experiment, 200 g of feed was used and the reactor was flushed with N_2 with a flow of 10 L/min for anoxic environment. The combustion was carried out from the ambient temperature to the final reaction temperature of 500°C at a heating rate of 10°C/min. Once the reaction temperature reached 500°C, the reactor was kept for 20 min. After the pyrolysis, the reactor was purged by N_2 for 1 h. The pyrolysis plant is described schematically in Supporting Information Fig. S1.

For identification and quantification of the bio-oil components, a GC-MS (5975C, Agilent Instruments, USA) system was employed. For GC-MS analysis, bio-oil was initially well mixed and 0.4-μL aliquot of the mixed bio-oil sample was manually injected with a 10-μL syringe into a HP-5MS (30 m × 0.25 mm diameter, 0.25-μm film thickness) capillary column.

Cell Culture and Bio-oils Treatment

Jurkat T cells (human lymphoma) were maintained in RPMI1640, and HepG2 (human liver carcinoma cells) were cultured in MEM (GIBCO), both supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37°C in a 5% CO_2 atmosphere. HepG2 cells were used between 5 and 20 passages. Both types of cells were exposed to bio-oils (rice oil and oak oil in DMSO) that was mixed with

cell-specific media at different concentrations, ranging from 0.001% to 0.1% (for Jurkat T cells) or up to 0.25% (for HepG2 cells). Treated and control (without bio-oil) cells were incubated for 24 h and then harvested for the next experimental steps.

Cytotoxicity and Cell Viability

Cytotoxicity of each bio-oil was determined by EZ-Cytox cell viability assay kit (Daeil Lab Service, Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. Approximately, 5×10^4 cells/well (for Jurkat T cells) and 1×10^4 cells/well (for HepG2 cells) were seeded in 96-well plates 24 h prior to treatment and exposed to two different bio-oils at the concentrations mentioned in the previous section (Cell Culture and Bio-oils Treatment) for another 24 h; 10 μL of EZ-Cytox reagent was added to each well including treated and control (without bio-oils). Absorbance (OD450) was detected at 450 nm after 3 h of incubation at 37°C. Appropriate blanks were used for each concentration to validate the absorbance.

Cell viability was measured using the standard trypan blue (Invitrogene, USA) staining method, and the total numbers of stained and unstained cells were counted using a hemocytometer. Three separate experiments were performed for all concentrations in triplicate, and EC_{50}s were calculated by using four parametric logistic equations.

Apoptosis Assay

Jurkat T cells were (grown on six-well plates at 1×10^6 cells/well for 24 h prior to treatment) treated with two bio-oils at the concentrations of 0.001%, 0.005%, 0.01%, and 0.05% for 24 h. Apoptosis was determined by flow cytometry (Cell Lab Quanta™ SC, Beckman Coulter, Fullertonm, USA) after staining with annexin V/PI (Annexin V-FITC apoptosis kit, BioVision, USA). HepG2 cells were grown on poly-L-lycine coated cover slips for 24 h at 5×10^5 cells/well and treated with bio-oils at concentrations of 0.001%, 0.005%, and 0.01% for 24 h. Apoptotic body was identified using the fluorescence observed via a Leica DM IL microscope, with images obtained using a Leica DCF 420C camera (Leica, Germany) after staining with Hoechst 33342 (Sigma-Aldrich, USA).

Comet Assay

Jurkat T cells (1×10^6 cells/well) and HepG2 cells (5×10^5cells/well) were seeded in six-well plates before 24 h of treatment. Both the bio-oils were exposed to Jurkat T cells at concentrations of 0.001%, 0.005%, 0.01%, and 0.05%, whereas HepG2 cells were treated with 0.005%, 0.01%, 0.05%, and 0.1% of both bio-oils for 24 h.

After the treatments, alkaline comet assay was performed following the method described previously (Park et al., 2008). The slides were stained with 50 μL of ethidium bromide (5 μg/mL) and analyzed using a fluorescence microscope (Leica DM IL, Leica, Germany) at 40× magnification. Approximately, 50 cells per slide (three slides per treatment) were examined. DNA damage was expressed as the tail extent moment using an image analysis by the Komet 5.5 software (Kinetic Imaging Ltd. UK).

Maintenance of *C. elegans* and Treatment of Bio-oils to Wild-type and Mutant *C. elegans*

C. elegans were grown in Petri dishes on nematode growth medium (NGM) and fed with *Escherichia coli* (OP50) according to standard protocol (Brenner, 1974). Worms were incubated at 20°C and the young adults (3-days old) from an age-synchronized culture were used in all the experiments. Wild-type (N2) and all mutant strains were provided by the Caenorhabditis Genetics Center (www.CGC.org) at the University of Minnesota.

The survival of the wild-type and mutant worms were assessed after 24 h exposure at different concentrations of both types of bio-oils as described previously (Roh et al., 2006). In brief, survival was determined by counting the number of live and dead individuals under a dissecting microscope while probing the worms with a platinum wire. Wild-type (N2) *C. elegans* was exposed to rice oil at concentrations of 0.5%, 1%, and 2%, whereas oak oil treatments were performed at concentrations of 0.25%, 0.5%, and 1% mixed in K-medium. LC_{50}s were derived through Probit analysis.

A wide range of mutant screening tests were performed at 1% of rice oil and 0.5% of oak oil. Three separate experiments were performed for all strains and concentrations each with triplicate.

Data Analysis

Significance of differences among and between treatments were determined using one-way analysis of variance (ANOVA) followed by a post-hoc test (Tukey, $p < 0.05$) in SPSS 12.0 KO (SPSS Inc., Chicago, Il, USA), and graphs were prepared in SigmaPlot (Version 12.0).

RESULTS AND DISCUSSIONS

This study is important not only in highlighting the production and biochemical composition of two pyrolysis oils, rice oil and oak oil, but also in presenting their detailed toxicological potentialities, i.e., cytotoxicity, genotoxicity, and comparative toxicity using *in vitro* and *in vivo* systems which in turn could help in proper risk assessment. To best of our knowledge, this is the first report to use *C. elegans* as a model system for bio-oil toxicity evaluation.

TABLE I. Analysis of two bio-oil, rice-oil and oak-oil, components by GC-MS

	Area (%)	
Components	Oak	Rice straw
Hydroxyacetic acid	2.2	n.d.
Formaldehyde	0.2	n.d.
Methanol	1.8	n.d.
Formic acid	n.d.	0.5
Methyl acetate	4	0.8
Acetic acid	35	35.1
Hydroxyacetone	6.3	10.7
Propanoic acid	0.5	3.7
Propenoic acid	0.1	n.d.
Pyridine	n.d.	0.24
Furfural	3.4	4.8
Furfuralcohol	0.5	1.3
2-methyl-2-cyclopentenone	0.3	0.6
1-(2-furanyl)-ethanone	0.2	0.6
Dihydro-2(3H)-furanone	0.3	0.8
2(5H)-furanone	0.5	n.d.
1,2-cyclopentanedione	1.6	0.9
5-methyl-2(3H)-furanone	0.1	n.d.
5-methylfurfural	0.7	0.4
1-(acetyloxy)-2-butanone	0.2	n.d.
3-methyl-2(5H)-furanone	0.2	n.d.
3-methyl-2-cyclopentenone	n.d.	0.7
Phenol	0.5	1.6
Corylon	1.7	1.8
2,3-dimethyl-2-cyclopentenone	0.3	0.4
o-Cresol	0.5	0.3
m-Cresol	0.6	0.8
Guaiacol	0.7	1
2,6-xylenol	0.2	n.d.
Maltol	0.5	n.d.
3-ethyl-2-hydroxy-2-cyclopenten-1-one	0.4	0.6
2,4-xylenol	0.3	n.d.
2-ethyl-phenol	n.d.	0.4
Creosol	0.3	n.d.
Pyrocatechol	3.1	1.2
5-hydroxymethylfurfural	0.2	n.d.
3-methoxy-pyrocatechol	0.9	0.3
2-methylresorcinol	1	0.1
Trimethylhydroquinone	0.2	n.d.
4-ethylguaiacol	0.2	n.d.
Resorcinol	0.3	n.d.
Hydroquinone	n.d.	0.3
Methylhydroquinone	n.d.	0.3
Syringol	1.2	0.7
Vanillin	0.2	0.1
Levoglucosan	n.d.	0.7
Homocatechol	1.1	n.d.
2,5-dimethylhydroquinone	0.3	n.d.
Vanillic acid	0.5	n.d.
Eugenol	0.3	n.d.
Syringaldehyde	0.1	n.d.
Acetosyringone	0.2	n.d.

n.d.: non-detected.

Characteristics of Bio-oils

The two bio-oils were produced from two most widely available feedstocks, rice straw and oak sawdust, and the organic components of bio-oils were characterized by GC-MS (Table I). The number of compositional chemicals was higher in oak oil (45) than in rice oil (30). It was found in GC-MS analysis that there was a high similarity in the composition of both oils (Table I). Among the major components, acetic acid, methyl acetate, furfural, furfuralcohol, corylon, phenol, pyrocatechol, propanoic acid, 5-methylfurfural, 1,2-cyclopentanedione, syringol, hydroxyacetone, and guaiacol were most common in both bio-oils. A distinguishable difference in concentration between the two bio-oils was found in methyl acetate, hydroxyacetone, propanoic acid, pyrocatechol, 2-methylresorcinol, etc. However, there were certain commercially important chemicals that differ significantly between two pyrolysis oils. It highlights the importance of feedstock variability on bio-oil quality under the same reactions conditions, as rice straw and oak wood differ in their cellulose, hemicellulose, and lignin content. Acetic acid, which is present most abundantly in both bio-oils, is usually present in high concentration in pyrolysis oils (Imam and Capareda, 2012) and could be isolated from bio-oils without any great expense, i.e., deacetylation (Vitasari et al., 2012). Another most common compound was furfural, which is a useful organic reagent for the production of medicines, resins, food additives, fuel additives, and other special chemicals (Hu et al., 2012). Hydroxyacetone, methyl acetate, pyrocatechol, and levoglucosan were present at different rates depending on feedstock type.

Notably, the available reports showed that most of the compositional chemicals of bio-oils possess hazardous potentiality and could cause acute to chronic toxicity in animals, ecotoxicity model species, and humans (Supplemental material Table S1). Therefore, for the sake of environmental health and safety assessment, we conducted thorough toxicity (acute 24 h) studies in both *in vitro* and *in vivo* model systems after these two new bio-oils exposure. It is important to note that most of the studies relating bio-oil analysis use semi-quantitative analysis results or area percentage of GC/FID or GC/MS analysis, although it is common to use the exact dosage (usually expressed as molar or metric units) in toxicology study. It is technically difficult to express each component in bio-oil in molar or metric units, because bio-oil contains a variety of components (Table I), some of which cannot be easily available commercially.

Effect of Bio-oils on Cell Viability and Cytotoxicity *In vitro* Systems

The cell viability assays demonstrated a dose-dependent increase in cytotoxicity [Figs. 1(A) and 2(A)] and decrease in cell viability [Figs. 1(B) and 2(B)] in both cell types—Jurkat T cells (Fig. 1) and HepG2 (Fig. 2) after 24-h

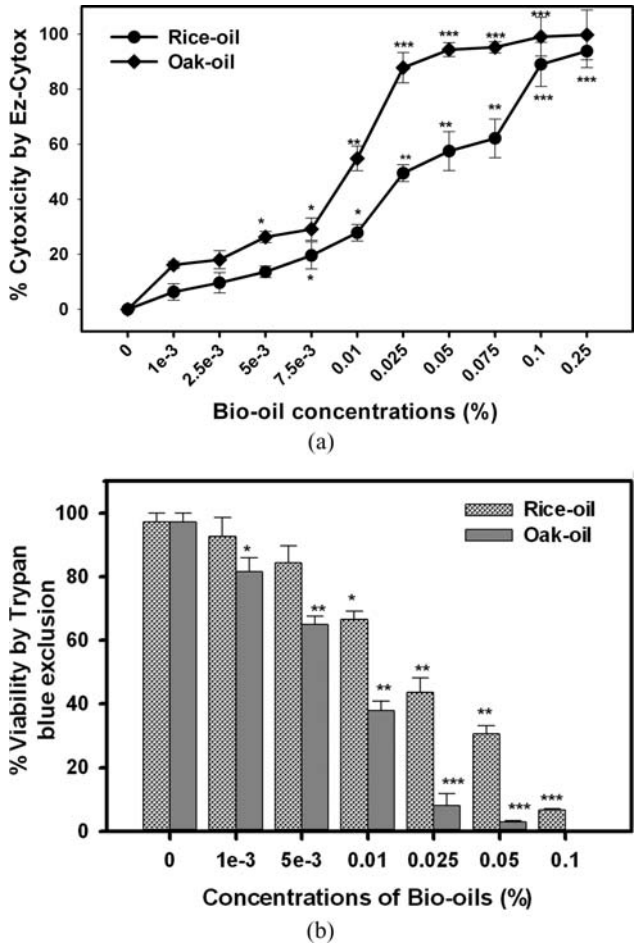

Fig. 1. Effect of 24-hr exposure of bio-oils (rice oil and oak oil) on cytotoxicity (A) and viability (B) of Jurkat T cells. The results are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ compared with untreated control (one-way ANOVA).

exposure to bio-oils. Effective concentrations (ECs) were-analyzed by using the four-parametric logistic equation from trypan blue exclusion viability results (Table II). Oak oil showed higher cytotoxicity compared with rice oil, and Jurkat T cells displayed higher sensitivity than HepG2 cells. The EC_{50}s of oak oil were about 0.008% for Jurkat T cells and 0.044% for HepG2 cells and in comparison, the rice oil EC_{50}s are about 0.048% in case of Jurkat T cells and 0.136% for HepG2 cells. The differential susceptibility of the cultured cells actually lies in the nature of the cell systems: Jurkat T, a suspended cell line, whereas HepG2, an attached cell line, which in turn govern the bio-oils mediated toxicity to the corresponding system.

We assessed cytotoxicity with two independent assays—Ez-cytox and trypan blue exclusion—for accurate validation of the findings as was suggested by Kong et al. (2011) for new nanomaterials toxicity evaluation. In general, trypan blue exclusion assay, based on membrane integrity, is a traditional method of viability assessment, whereas EZ-Cytox assay, which exploited metabolic activity and mitochondrial function, is a new cytotoxicity assessment kit but has also been used by several studies (Kang et al., 2010; Kwon et al., 2010). Notably, these two cytotoxicity assays do not work on the same principle, which might be the reason behind the differential response of the assays toward the same dose of bio-oils.

Bio-oils Induced Apoptosis

Late apoptosis phenomena were much more prevalent than early apoptosis with a clear dose dependency in bio-oils exposed-Jurkat T cells [Fig. 3(A)] detected with flow cytometry after annexin V-FITC/PI staining. The two distinct modes of cell death—apoptosis and necrosis—were distinguished by morphological and bio-chemical features. Annexin V-FITC/PI staining of bio-oils-treated Jurkat T cells resulted in an increase in annexin V^+/PI^- (early apoptosis), annexin V^+/PI^+ (late apoptosis) in comparison to a

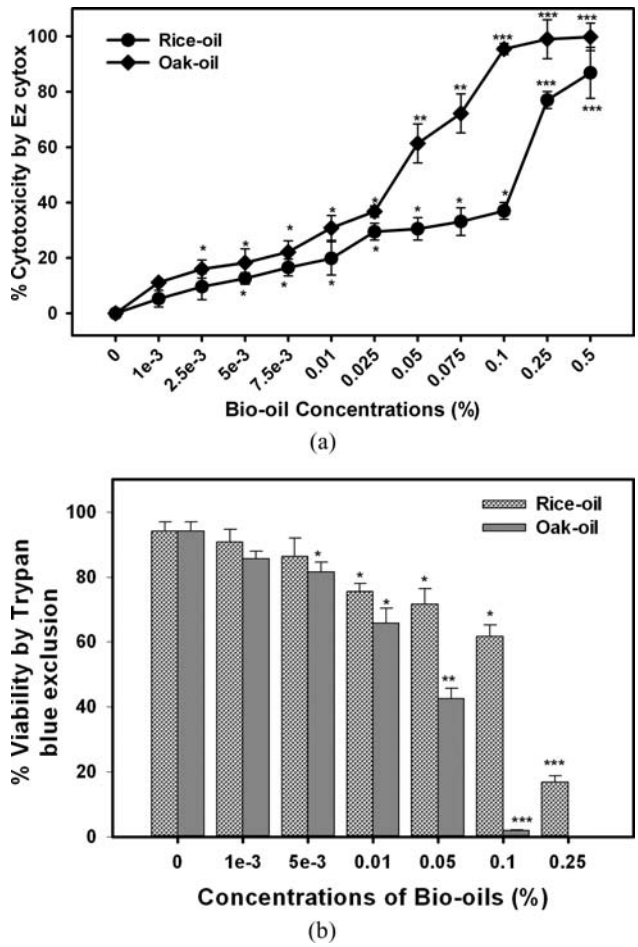

Fig. 2. Effect of 24-hr exposure of bio-oils (rice oil and oak oil) on cytotoxicity (A) and viability (B) of HepG2 cells. The results are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ compared with untreated control (one-way ANOVA).

TABLE II. Estimation of 24-h lethal concentrations of bio-oils to in vitro cultured cell systems[a]

Cultured Cell System	24-h ECs	Oak Oil (%)	Rice Oil (%)
Jurkat T cells	EC_{10}	0.0003	0.002
	EC_{50}	0.008	0.048
	EC_{90}	0.016	0.096
HepG2	EC_{10}	0.0006	0.0008
	EC_{50}	0.044	0.136
	EC_{90}	0.089	0.272

[a]EC, effective concentration; EC_{10}, 10% effective concentration; EC_{50}, 50% effective concentration; EC_{90}, 90% effective concentration.

negligible increase in annexin V^-/PI^+ (necrosis) and decrease in annexin V^-/PI^- (viable) cells compared to the control, indicating apoptosis as a possible mode of cell death [Fig. 3(A)]. Prevalence in late apoptosis might be an effect of chosen time point of exposure, i.e., 24 h. For further confirmation, we examined the HepG2 cells stained with Hoechst 33342 after exposure to bio-oils (0.001%, 0.005%, and 0.01%) for 24 h and found nuclear condensation and fragmentation [Fig. 3(B)], which is another well-known morphological hallmark of apoptosis (Zhivotovsky, 2004). Similar to viability, apoptosis (late apoptosis) was more profound in oak oil-exposed cells.

Genotoxic Potentiality of Bio-oils

DNA damage (single- and double-strand breakage, DNA–DNA crosslinks, DNA-protein crosslinks, and DNA adducts) in a somatic cell might result in a mutation, which in turn leads to malignant transformation (cancer) and the DNA

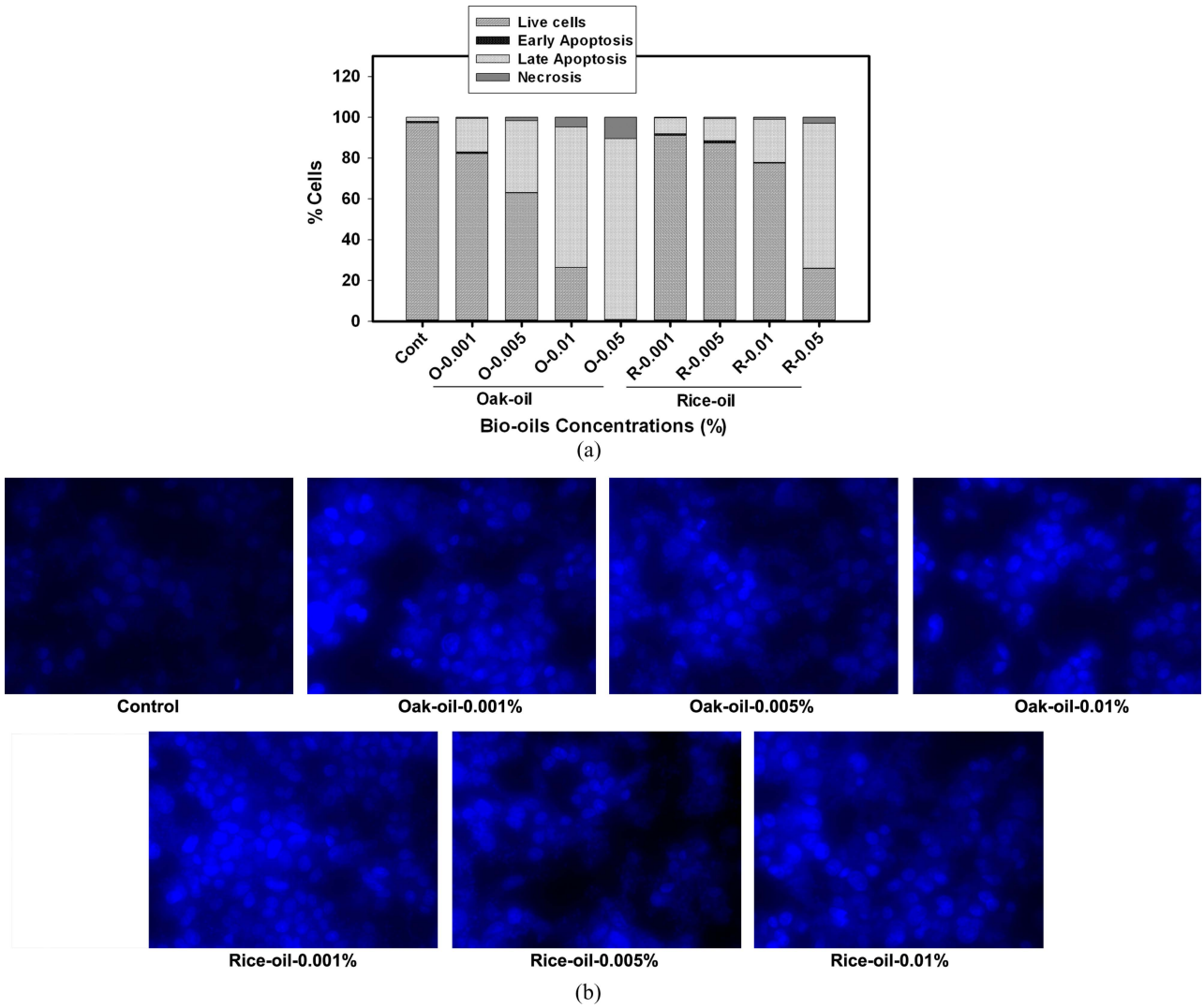

Fig. 3. (A) Apoptosis in Jurkat T cells exposed to bio-oils at different concentrations (0.001%, 0.005%, 0.01%, and 0.05%) for 24 h using Annexin V/PI staining followed by flow-cytometric analysis. (B) Apoptosis (condensed and fragmented nuclei) in HepG2 cells exposed to bio-oils at different concentrations (0.001%, 0.005%, and 0.01%) for 24 h using Hoechst 33342 staining followed by microscopic analysis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

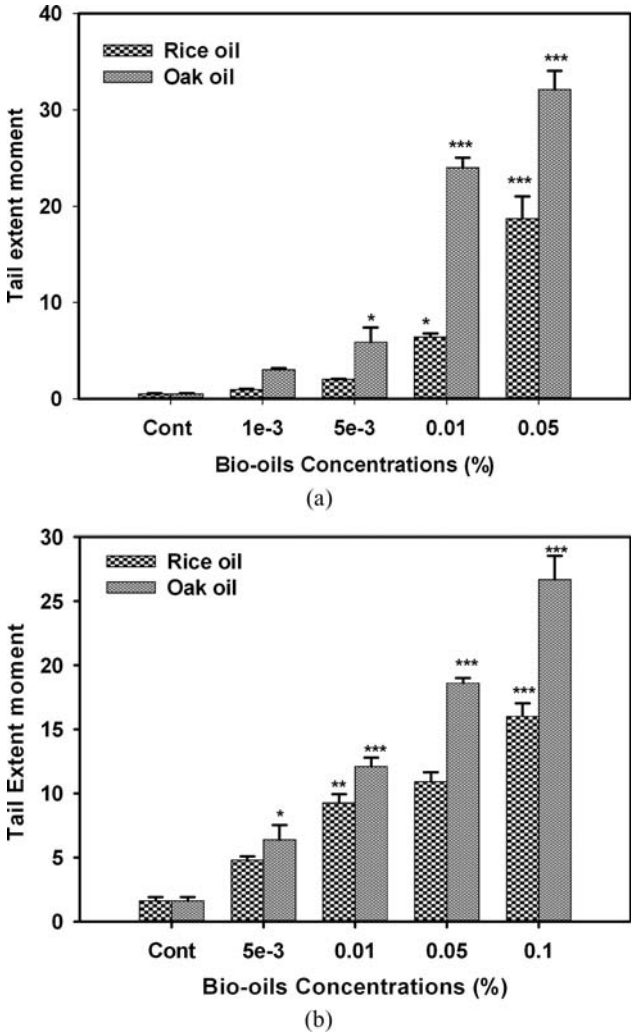

Fig. 4. Genotoxicity of bio-oils to Jurkat T and HepG2 cells. (A) Plot of comet tail moments of 24-h bio-oils exposed Jurkat T cells indicated increasing DNA damage with increasing bio-oils concentration. (B) Plot of comet tail moments of 24-h bio-oils treated HepG2 cells indicated increasing DNA damage with increasing bio-oils concentration. Data represent mean ± SEM ($n = 50$) *$p < 0.05$ and ***$p < 0.0001$ compared with untreated control (ANOVA).

strand break, especially double strand break, is considered as the most detrimental one (Jackson, 2002) among all the types of DNA lesions. Hence, detection of DNA strand breaks is a sensitive marker of genotoxic damage potential and pre-mutagenic lesions. Among the available DNA strand break and subsequent genotoxicity indicator tests, the comet assay is capable of detecting single as well as double DNA strand breaks and alkali labile sites even at low levels of DNA damage (Collins, 2004). In this study, we observed the genotoxic potential of bio-oils in Jurkat T [Fig. 4(A), Supporting Information Fig. S2] and HepG2 cells [Fig. 4(B), Supporting Information Fig. S3] in the alkaline comet assay, and the significant induction ($p < 0.001$) of comet tail extent moment confirms the genotoxic potentialities of bio-oils. These results are in agreement with the findings of our previous study (Park et al., 2008) in which the authors observed DNA lesions in cultured L5178Y cells after exposure to upper and lower phase of bio-oils produced from radiata pine. As was expected, oak oil possessed higher genotoxic potentiality than rice oil (Fig.4 and Supporting Information Figs. S2 and S3) in both the cell lines.

Survival of Wild-type (N2) *C. elegans*

Acute toxicity of bio-oils (oak oil and rice oil) on *C. elegans* was investigated using mortality as endpoint and percentage of survival at different concentrations of bio-oils, which are displayed in Figure 5. In case of rice oil exposure, a dose-dependent decrease in viability was evident and on the contrary, a sharp decrease of viability was observed at the concentration of 0.5%, and all worms were dead at a dose of 1% of oak oil (Fig. 5). Lethal concentrations (LCs) were analyzed by using Probit (Table III). The LC_{50} (24 h) of oak oil in *C. elegans* was estimated as 0.492% and comparatively 0.991% for rice oil. The bio-oils behave in the same way for both *in vitro* and *in vivo* model systems, and likewise in the *in vitro* cultured cell system oak-oil was also found to be more toxic in *C. elegans*.

Oak oil consistently demonstrated much higher toxicity with respect to all the toxicity end points tested in all the model systems. Therefore, the difference between the toxic potentialities of bio-oils seems to be dependent on the raw material used as a source of bio-oils production.

C. elegans Mutants Screening

Further, to get an overview of the mechanism of toxicity posed by bio-oil we screened a series of available mutants at

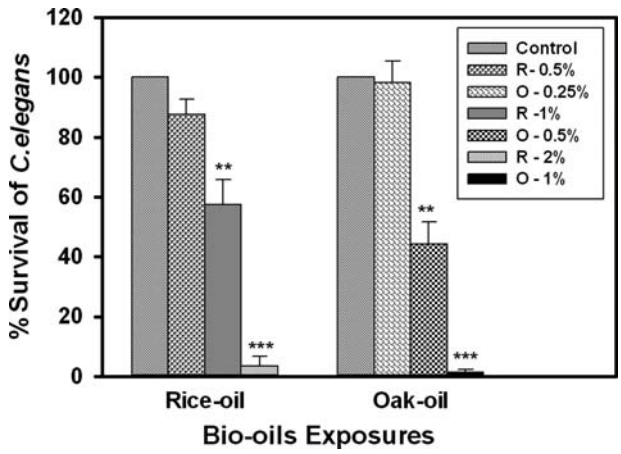

Fig. 5. Survival percentage of wild-type *C. elegans* due to bio-oils exposure at different concentrations of rice oils (R-0.5%, 1%, and 2%) and oak oil (O-0.25%, 0.5%, and 1%) for 24 h. Data represent mean ± SEM. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$ compared with untreated control (one-way ANOVA).

TABLE III. Estimation of 24-h lethal concentrations of bio-oils (rice oil and oak oil) in C. elegans[a]

24-h LCs	Oak Oil (%)	Rice Oil (%)
LC_{10}	0.341	0.535
LC_{50}	0.492	0.991
LC_{90}	0.683	1.441

[a]LC, lethal concentration; LC_{10}, 10% lethal concentration; LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration.

the dose of approximate LC_{50} values of both the bio-oils, i.e., 0.5% of oak oil and 1% of rice oil. The details of the mutant strains are presented in the supplemental material (Table S2). Survivals of mutants at those particular concentrations of bio-oils were calculated with reference to the wild-type strain (N_2) (Fig. 6). Almost all the mutants were found to be more sensitive than N_2, except *cep-1* and *ced-3* mutant strains. Among the sensitive strains *pmk-1* mutant was found as most sensitive where almost all the worms were dead at the LC_{50}s of N_2. The order of the sensitivity of the mutant strains are *pmk-1> daf-16, sir-2.1 > hif-1 > ctl-2 > cyp35a2 > mtl-2 > sod-3 > hsp16.2* (Fig. 6). The order of resistant strains are *ced-3 < cep-1*. The *mtl-2* and *sod-3* mutants are more sensitive to LC_{50} (of N2) of rice oil. In general, a similar pattern of responses were found within the same mutants exposed to both the bio-oils, i.e., more sensitive or resistant to oak oil than rice oil.

The results suggested that the genes, such as *pmk-1, daf-16, sir2.1, hif-1, and cyp35a2*, might have involvement in the protection to bio-oils as those defective gene mutants showed higher sensitivities than N_2, conversely, the gene, namely *cep-1* and *ced-3*, might be responsible for bio-oil toxicity as its defective mutant displayed higher resistance than N_2. In our previous study with silver nanoparticles, the gene *pmk-1*, an otholog of human p38MAPK and a key molecule in worms innate immunity (Troemel et al., 2006), was found to be involved in chemical stress response (Lim et al., 2012). As *pmk-1* deficient mutant was found to be the most sensitive one, so it would be worthy to postulate that bio-oils possibly exerted toxicity by affecting the worm's defense and immune system. The second most sensitive strains were found to be *sir-2.1* and *daf-16* mutants. The *C. elegans sir-2.1* has been shown to regulate nematode adult lifespan and aging via the insulin/IGF path-way transcription factor *daf-16* (Viswanathan et al., 2005) and subsequently dauer formation, longevity, fat metabolism, stress response, and innate immunity are regulated by the *daf-16*. So, the bio-oils might exert a toxic response through affecting life span. Further, the next level of sensitivities was found in *hif-1*, *ctl-2*, and *cyp35a2* mutant worms. The *C. elegans*' *hif-1* is required to survive in hypoxic environments (1% oxygen) and takes part in adult lifespan determination. The gene *ctl-2*, which encodes one of three *C. elegans* catalases, works as an antioxidant enzyme and *cyp35a2*, the *C. elegans* cytochrome P450s, mainly involves in the metabolism of endogenous and exogenous compounds, including xenobiotics, and is manifested to play an important role in oxidation-reduction processes. In particular, it has been reported that almost all cyp35 isoforms in *C. elegans* were moderately or strongly activated by different xenobiotics in a cyp450 gene expression–screening experiment (Menzel et al., 2005; Roh et al., 2006). Hence, the susceptibility of the *hif-1*, *ctl-2*, and *cyp35a2* mutants proved that bio-oils exposure is possibly responsible for oxidative stress, as well. On the contrary, the significant tolerance to bio-oils was evident in *cep-1* and *ced-3* mutant strains (Fig. 6). Both genes are known for execution of apoptosis, and deletion mutant strains of *cep-1* was found to be resistant to DNA damage-induced germ cell apoptosis (Derry et al., 2001), whereas loss of *ced-3* function is reported to cause transformation of cell fate and resistance to cell death (Ellis and Horvitz, 1986). Therefore, increased tolerance of these two mutants to bio-oils manifested that apoptosis was one of the main mechanisms in bio-oil mediated toxicity to worms, which indeed is the mode of cell death of bio-oils exposed *in vitro* systems.

Taken together, the underlying mechanisms of bio-oil toxicity were oxidative stress and impairment of the immune system, which ultimately caused death by affecting the lifespan of the worm. Although the mutant strains and the toxicity parameters tested in this study were rather limited in terms of full understanding of the toxic mechanism of bio-oils, these preliminary mutant screening tests evoked particular interest for further study of *pmk-1*, *sir2.1*, and *daf-16* sensitivities and *cep-1* resistance to bio-oils. Furthermore, the current study supports the idea that *C. elegans* could serve as an excellent alternative *in vivo* model system to identify toxicity of new materials, and mutant screening

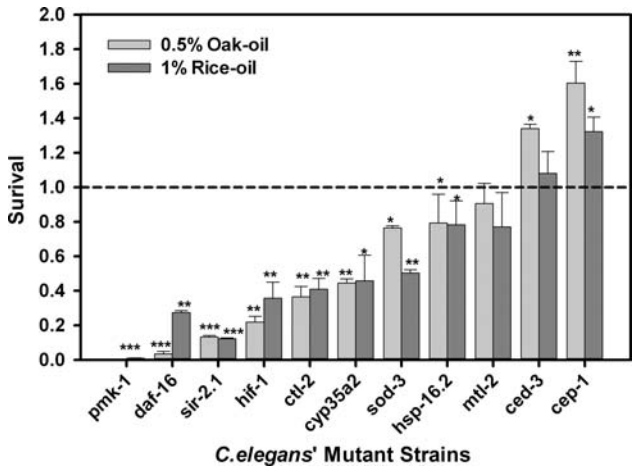

Fig. 6. Survival of different *C. elegans* mutant strains due to bio-oils exposure. Data are presented in arbitrary unit compared wild-type strain (N2)'s survival at the same concentrations of bio-oils (N2 survival = 1). Data represent mean ± SEM. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$ compared with untreated control (one-way ANOVA).

seems to be a promising tool for toxicological mechanism studies which in turn uphold the use of mutant strains as bio-indicators for a wide range of environmental chemicals (Chu et al., 2005; Roh et al., 2009).

Among the common chemicals (mentioned in the Characteristics of Bio-oils section) phenol, acetic acid, corylon, 5-methylfurfural, furfuralcohol, 3-methoxy-pyrocatechol, etc., might be responsible for the DNA damage potentiality of both the bio-oils, as these compounds are known to be mutagenic and/or carcinogenic (Table S1). The mode of toxicity of both the bio-oils was found to be almost similar in *C. elegans* mutant screening, possibly because of a high number of common chemicals possessed by both bio-oils. Besides human toxicity, some compounds are well known for their acute animal toxicity and ecotoxicity as well, for instance, furfural, phenol, furfuralcohol, dihydro-2(3H)-furanone (aquatic microbes and algae), etc. Some compounds caused target organ damage with repetitive exposure, while non-hazardous in acute toxicity tests (Table S1). The higher toxic potentiality of oak oil might be because of the presence of hydroxyacetic acid (developmental toxin), methanol (mutagenic, teratogenic), formaldehyde (carcinogenic, mutagenic, and possibly neurotoxic), homocatechol (carcinogenic), resorcinol (mutagenic), 5-hydroxymethylfurfural (mutagenic and carcinogenic), propenoic acid (suspected mutagen, teratogen, developmental, and male reproductive toxin), eugenol (hepatotoxic), maltol (cause apoptosis), 2(5H)-furanone (reproductive toxin), etc., which are unique in oak oil and not present in rice oil. These support the idea that the composition of the bio-oils mainly depends on the feedstock used, which in turn modulates the toxic potentiality (Cirad, BIOTOX, 2005; Cirad, 2006)

CONCLUSIONS

In summary, the components of the bio-oils from the pyrolysis of rice straw and oak sawdust were determined, and their cytotoxicity and genotoxicity were evaluated in Jurkat T and HepG2 cells. The cell lines exposed to bio-oils showed a decrease in cell viability, and the mode of bio-oils induced cell death was found to be apoptosis. Bio-oils also induced DNA damage, which was attested by comet assay. In addition, *in vivo* acute toxicity was measured by using *C. elegans* as a model system for better extrapolation to higher eukaryotes. Oak oil was found to be more toxic than rice oil in all the systems and in all toxicity end points tested. Moreover, several *C. elegans* mutants screening not only provided valuable insights into the mechanism of bio-oil induced toxicity but also led our future research direction. Most importantly, the toxic potentialities of bio-oils depend on the pyrolysis method and the feedstock used for the production. Notably, the results of this study possess great importance for environmental health and safety assessment as the bio-oils, rice-oil and oak-oil, have the potential to be used as bio-fuels or for the extraction of other commercial chemicals, for example, food additives. Furthermore, it is possible to determine the best-operating conditions to avoid or minimize the formation of toxic products from the bio-oils by comparing this study with other available bio-oils (production processes and feedstock). Above all, this study could be particularly useful for deducing the hazardous potential of the other bio-oils with similar chemical compositions and could successfully eliminate the time-consuming experimental steps to obtain their toxicological data.

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