Journal of Medicinal Plants Research Vol. 3(12), pp. 1092-1098, December, 2009
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875© 2009 Academic Journals

Full Length Research Paper

Evaluation of biological activities of *Rhaponticum carthamoides* extracts

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Accepted 13 November, 2009

Rhaponticum carthamoides **is an endemic medicinal plant of Siberian origin. Its roots tissue is well known as a rich source of biologically active compounds.** ***R. carthamoides*** **crude extracts were obtained with two different extraction methods (sonication and shaking) and three extraction solvents with different polarity (chloroform, methanol and water). All extract were screened for antioxidative activity (phosphomolybdenum assay, β-carotene bleaching test, DPPH and FRAP tests), cytotoxic (MTT assay) and mutagenic activities (modified Ames test, with the use** ***Vibrio harveyi*** **strains). All tested extracts were able to reduce molybdenum and inhibit about 85% of linoleic acid peroxidation. However, only water and methanol extracts reduced Fe^{+3} ions and scavenged DPPH free radical. On the other hand chloroform extracts indicated minor toxicity against all tested cell lines. None of the tested extracts showed mutagenic activity.**

Key words: Antioxidative activity, cytotoxic activity, mutagenic activity, *Rhaponticum carthamoides.*

INTRODUCTION

Rhaponticum carthamoides (Willd.) Iljin. (*Leuzea carthamoides* D.C.) is an endemic, perennial medicinal plant of Siberian origin. Its roots, which have a novel type of pharmacological action classified as adaptogenic, are commonly used for medicinal purposes in Russia. *R. carthamoides* was widely used in folklore medicine (usually roots) to treat overstrain and common weakness after illnesses. *R. carthamoides* is usually found in high-mountain meadows, in tundra brushwood and in the glades of coniferous forests (Petkov et al., 1984).

A number of active compounds have being isolated from either its leaves or roots. These include flavonoids (Sharaf et al., 2001), organic acids (Yance, 2004) and phytoecdysteoids (Baltayev, 1995; Baltayev et al., 1997). Phytoecdysteroids of which 20-hydroxyecdysone (20E) is the most abundant, exert immunomodulatory (Trenin and Volodin, 1999), adaptogenic and anabolic effects on vertebrates (Petkov et al., 1984; Slama et al., 1996). *R. carthamoides* extracts (RCEs) are also capable of stimulating the total protein content in liver, pancreas, thymus and adrenal glands (Todorov et al., 2000).

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Before now, attention has being laid on the activity of *R. carthamoides* root and rhizome extracts (Bespalov et al., 1992; Petkov et al., 1984; Todorov et al., 2000). However, collecting plant material in such a manner inevitably results in eliminating the plant. Currently more interest is on the investigation of the aerial part (Kokoska et al., 2002; Miliauskas et al., 2004) since it provides the annual supply of plant material, without the necessity to renew part or the whole plantation.

The goal of the present study was to describe the biological activity (antioxidative, cytotoxic and mutagenic) of different extracts obtained from *R. carthamoides* aerial parts.

MATERIALS AND METHODS

Plant material and extraction procedures

Plant material grown in Syberia (dried leaves of *R. carthamoides*) were provided by FITOSTAR™. One gram of plant material was grounded in a mortar and then 20 ml of a suitable solvent – water, methanol or chloroform (POCh) were added. The suspension was either sonicated for 30 min (PolSonic) or shaken for 24 h (horizontal shaker, JW Electronic WL 2000). Subsequently the suspension was filtered twice through the double Whatmann 1 blotting paper (Merck) and the solvent was evaporated to dryness at an ambient temperature. Residues obtained after extraction were weighed and

dissolved in methanol.

Cell lines and culture conditions

HeLa and HL-60 cell lines were obtained from the Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology. HEK and U937 cell lines were obtained from the Division of Cell Biology, Intercollegiate Faculty of Biotechnology UG & MUG, Gdansk. Cells were grown in minimum essential medium (MEM), Dulbecco's modified eagles medium (DMEM) or Roswell park memorial institute (RPMI) (Sigma Aldrich) medium (in case of HeLa, HEK and both suspension cell lines respectively), supplemented with 10% foetal bovine serum (FBS) (GIBCO^{TM}), antibiotics (GIBCO^{TM}), 1% L-glutamine (GIBCO^{TM}) and non-essential amino acids (GIBCO^{TM}). Cells cultures were incubated at 37°C in an atmosphere of 5% CO_2 (Heraeus Instruments). Cells were subcultured every 3 – 4 days.

Antioxidant activity assays

Phosphomolybdenum method (PMA)

The procedure of Prieto et al. (1999) with minor modifications was followed in order to estimate the total antioxidant activity of crude chloroform, methanol and water of *R. carthamoides* extract (RCE). Briefly, 100 µl of extract samples (2.5 mg RCE/ml) were mixed with 900 µl of the reaction mixture (0.6 M sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molibdate). The test tubes were incubated for 90 min at 95°C and cooled to room temperature. Subsequently the samples were diluted 20 times and finally absorption was measured at a wave length of 695 nm (Beckman DU□640 Spectrophotometer).

Antioxidants used for comparative purposes were α-tocopherol (MP Biochemicals Inc.), ascorbic acid (Sigma Aldrich) and butylated hydroxytoluene (BHT) (MP Biochemicals Inc.). A standard curve was constructed for ascorbic acid in order to estimate the percentage of molybdenum reduced by tested extracts.

β-Carotene bleaching method (β-CB)

An antioxidant test using the β-carotene-linoleate model system was performed as described by Taga et al. (1984) with modifications proposed by Zhang and Hamauzu (2003). 0.03% w/v β-carotene (Fluka) solution in chloroform was prepared and mixed with linoleic acid (Fluka) and Tween 20 (Koch Light Laboratories Ltd.) (3 ml: 40 µl : 400 µl). Tween 20 was incubated in a water bath at 50°C prior to use. Subsequently, chloroform was evaporated and 100 ml of water, preferably oxygenated, were added to the emulsion left. 3 ml emulsion aliquots were mixed with 200 µl RCE or control antioxidants (2.5 mg/ml). Test tubes were incubated at 50ºC for 120 min. Sample absorbance was measured at 470 nm at time points 0 and 120 min. Antioxidant activity (AA) was calculated according the formula by Hidalgo et al. (1994).

$$AA = 100 \times [1- (A_0-A_t) / (A^c_0-A^c_t)]$$

Where, A_0, A^c_0 = absorption measured at the beginning of incubation for the analyte and control, respectively; A_t, A^c_t = absorption measured after 120 min incubation for the analyte and control, respectively. RCEs activities were compared with known antioxidants of α-tocopherol, ascorbic acid and BHT and 20E (Sigma Aldrich; min. 95%, purified by HPLC).

Iron reducing (FRAP)

RCEs ability to reduce iron ($Fe^{+3} \rightarrow Fe^{+2}$) was estimated with the use of FRAP (Ferric Reducing Capacity of Plasma) (Benzie and Strain, 1996) with minor modifications. Working solution consisted of 0.3 M acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine; Sigma) in 40 mM HCl and 20 mM $FeCl_3$ $6H_2O$, in the following proportions 10:1:1. Serial dilutions of 30 µl RCE, 20E or control antioxidants (see above) were prepared in microplates and mixed with aliquots (170 µl) of working solution. Concentration range of RCE, 20E and antioxidants was 0.15 – 150 µg/ ml. After 20 min incubation absorption was measured at 593 nm (Wallac Victor, 1420 Multilabel Counter).

A standard curve for ascorbic acid was constructed in order to estimate the percentage of iron reduced and to determined the IC_{50} value, that is, concentration of RCE at which 50% of ferric ions were reduced.

2,2-Diphenyl-1-pichydrazyl (DPPH) assay

Radical scavenging activity (RSA) was determined using a DPPH assay as described by Konczak-Islam et al. (2003) with slight modifications. Serial dilutions of chloroform, methanol and water extracts, antioxidants and 20E (concentration range 1 – 1000 µg/ml) were pipetted into 96-well plate. The stock solution of 2,2-diphenyl-1-pichydrazyl (DPPH) stable free radical was prepared in methanol. Aliquots of DPPH were added into the wells to the final concentration of 0.1 mM. After 2 min incubation, absorption was measured at the wavelength of 517 nm (Wallac Victor, 1420 Multilabel Counter). RCEs activity was compared with α-tocopherol, ascorbic acid and BHT as above. The results were calculated using the following formula:

$$RSA\ (\%) = [(Abs_{517control} - Abs_{517sample})/ Abs_{517control}] \times 100$$

RSA = radical scavenging activity; Abs_{517} = absorption at 517 nm; control= non-reduced DPPH.

Cytotoxic activity - Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Cells were harvested (with the use of trypsine (Sigma) in case of the adherent HeLa cell line), counted (Reichert hemocytometer) and aliquots of 100 µl cell suspension (2 x 10^5 cells/ml) were pipetted into 96-well plates. The plate was incubated at 37°C at an atmosphere of 5% CO_2. On the following day plant extracts (final concentrations 5 – 50 µg/ml in reaction mixture) or 20E (final concentration 50 µg/ml) were applied into the wells. 3 µl of pure methanol was applied as a control (aliquot of the analytes). Cells were incubated with the extracts or 20E for 24 h in conditions as described above.

At 2 h prior to culture termination 10 µl of MTT (Sigma) solution (5 mg/ml PBS, pH = 7.4) was pipetted into the wells. Following incubation, the medium was aspirated and formazan crystals were dissolved in 99.9% dimethyl sulfoxide (DMSO; Sigma). The colour intensity was measured spectrophotometrically at the wavelength 570 nm with a multiplate reader (Wallace Victor, 1420 Multilabel Counter). The test was performed in duplicates, in three independent series. The results were calculated using the following formula:

$$Viabililty\ (\%) = [(Abs_{570sample} - Abs_{570blank})/(Abs_{570control} - Abs_{570blank})] \times 100$$

Abs_{570} = absorbance at λ 570 nm; control = non-treated cells.

Mutagenic activity

Vibrio harveyi BB7 and BB7M strains were obtained through the courtesy of the Department of Molecular Biology, University of Gdansk. BB7 is a wild type strain; BB7M is a genetically modified

strain, bearing pAB91273 plasmid with resistance to chloramphenicol, expressing *mucA* and *mucB* genes (Czyz et al., 2000b). The latter characteristics resulted in an increase in the efficiency of an error-prone DNA repair system and elevated frequency of mutations, here also those associated with neomycin resistance.

V. harveyi were grown on solid BOSS medium (3% NaCl, 1% peptone, 0.3% yeast extract, 0.1% glycerol; pH = 7.3) in 30°C. In the case of the BB7M strain the culture medium was supplemented with 34 mg/ml chloramphenicol as a selection agent. The assay for mutagenic activity of *R. carthamoides* extracts was performed as described by Czyz et al. (2000b). Briefly, an overnight culture was diluted to an OD_{575} = 0.1 and aliquots of 100 µl (ca. 4 x 10^6 cfu) were spread on BOSS medium, containing 50 µg/ml extracts and 50 µg RCE/ml neomycin. The highest concentration of RCE, which does not influence the bacteria viability, was used. Pure methanol and 1.5 µg/ml sodium azide instead of the extract served as negative and positive controls, respectively. Plates were incubated 6 - 7 days, then bacterial colonies were counted and the percentage of neomycin-resistant mutants was calculated.

Statistical analysis

All experiments were performed in three independent series and the results were expressed as the mean value ± S.D. In the case of the mutagenic activity assay the ANOVA test was performed and $P < 0.05$ value was considered as statistically significant.

RESULTS AND DISCUSSION

Optimization of the extraction methods

R. carthamoides extracts were prepared using two extraction methods and three solvents, differing in polarity. The extraction yield depends on the solvent used and extraction method applied (Table 1). The efficiency of the water extraction was about twelve times higher in the case of chloroform. However it should be noted that together with the active compounds some other polar cell components like polysaccharides or proteins might have been extracted. Extraction yield also differed with respect to the method applied. Higher results were consistently obtained in the case of sonication and therefore this method should be recommended for further work, since it is more efficient and less time consuming than shaking.

Table 1. Yields of extract obtained from dried *R. carthamoides* tissue using sonication or shaking extraction methods with different solvent.

Extracting solvent	Yield of extract (mg/g DW)	
	Sonication	Shaking
Chloroform	12.72 ± 1,68	8.33 ± 0.44
Methanol	67.30 ± 3.07	41.32 ± 4.06
Water	123.82 ± 30.65	107.55 ± 7.28

Antioxidant activity

Antioxidant activity of *R. carthamoides* extracts was determined with the use of four methods, differing in reaction mechanism and environmental polarity. Diversification is necessary to obtain credible results, since they may be influenced by analyte polarity or its mode of action. For instance compounds that are able to prevent oxidation through non-radical reaction mechanism may be weak radical scavengers. This was demonstrated by Mansouri and co-workers (Mansouri et al., 2005) for cinnamic acids and their benzoate analogues. However, unlike isolated compounds, the observed effect seen in crude extracts or fractions with a mixture of components could be as a result of the interaction between the components and therefore drawing unambiguous conclusions may be complicated. The four methods used for the assay of antioxidant activities are discussed below.

Phosphomolybdenum method (PMA)

It is a quick, cheap and reproducible test, allowing the measurement of the antioxidant capacity of an analyte. In the presence of a reducing agent, reduction of phosphormolybdate acid to phosphomolybdate blue ($Mo^{+6} \rightarrow Mo^{+5}$) took place. The UV/visible spectrum of this compound has a characteristic maximum at 695 nm (Prieto et al., 1999). This method had been used successfully to determine the antioxidant activity of plant extracts (Konyalioglu et al., 2005) and isolated compounds for example, flavidin (Jayaprakasha et al., 2004).

RCEs were able to reduce molybdenum and the solvent used for the extract preparation seems to be of margin importance – all extracts' activity is of the same order of magnitude. The activity of RCEs is similar to the activity of BHT, however about 2 and 10 times lower that that of α-tocopherol and ascorbic acid, respectively (Table 2).

β-Carotene bleaching method

It was based on the coupled oxidation of β-carotene and linoleic acid. Oxygen present in the air or water reacts with unsaturated fatty acids (here: linoleic acid) forming peroxides. Peroxides, even in small quantities, are able to initiate a radical chain reaction resulting in linoleic acid decomposition. Its degradation products oxidizes β-carotene and the solution color intensity decreases (β-carotene bleaching) (Taga et al., 1984). In this case antioxidant activity determination was carried out in an emulsion and not in an aqueous environment as in the phosphomolybdenum and FRAPS methods. This results in the accessibility of the substrate for the potential antioxidant, in favour for lipophilic compounds.

Analogically as in the phosphomolybdenum method, all extracts exhibited antioxidant activity of similar strength (Table 2), though in this case comparable with α-tocopherol and BHT and much higher than ascorbic acid. This may suggest either a different mechanism of action

Table 2. Comparison of antioxidant activity of *R. carthamoides* extracts.

Analytes	PMA[a] (% of reduced Mo)	δ-CB[b] (% inhibition of linoleic acid peroxidation)	FRAP[c] (IC_{50} µg/ml)	DPPH[d] (IC_{50} µg/ml)
RCE chloroform/sonication	5.8 ± 2.8	81.94 ± 9.1	>150	>1000
RCE chloroform/shaking	6.3 ± 1.8	85.18 ± 5.2	>150	>1000
RCE methanol/sonication	3.6 ± 2.6	95.34 ± 8.1	53 ± 2.6	38.33 ± 6.3
RCE methanol/shaking	4.4 ± 3.5	85.58 ± 0.7	48.3 ± 3.1	84.20 ± 3.6
RCE water/sonication	4.8 ± 2.7	85.67 ± 10.4	87.7 ± 4.6	158.30 ± 52.6
RCE water/shaking	4.6 ± 2.5	91.47 ± 1.5	56 ± 2.6	139.97 ± 28.9
α-tocopherol	16.0 ± 3.8	98.11 ± 8.7	20.9 ± 2.8	10.78 ± 3.6
scorbic acid	66.1 ± 14.7	36.68 ± 0.2	3.9 ± 0.5	5.60 ± 4.7
BHT	6.5 ± 3.5	95.23 ± 21.5	18.5 ± 4.2	140.95 ± 42.1
20E	Not tested	21.12 ± 20.3	>150	>1000

[a]Antioxidant activity of RCEs monitored with the phosphomolybdenum assay.
[b]Antioxidant activity of RCEs monitored by δ-carotene bleaching test.
[c]Antioxidant activity of RCEs monitored by FRAP assay; IC_{50} = ❍g of analyte 1 ml of reaction mixture required for 50% Fe^{+3} ions reduced.
[d]Antioxidant activity of RCEs monitored by scavenging DPPH radicals; IC_{50} = ❍g of analyte in 1 ml of reaction mixture required for 50% radicals scavenged.

or influence of the reaction environment polarity. Ascorbic acid in the phosphomolybdenum test, exhibited 8.8 and 4.1 times higher reducing capacity than BHT and α-tocopherol, respectively and more than 9 times higher than RCEs. It is also worth noting that 20E exhibited very weak antioxidant potential.

FRAP assay

It was first designated to measure the ability of plasma to reduce ferric ions and therefore estimate its total reducing capacity (Benzie and Strain, 1996). However currently it is commonly applied for different materials and therefore is rather referred to as ferric reducing antioxidant power (Szollosi and Varga, 2002; Bahorun et al., 2004). Ferrous (Fe^{+2}) ions in the presence of TPTZ form a complex (λ_{max} = 593 nm), which results in the development of an intense blue colour. It should be noticed, that this test has some limitations – for instance glutathione and other antioxidants with –SH groups exhibit very low activity.

Only methanol and water RCEs showed the capacity to reduce ferric ions and their activity was only about 2.5 times lower than that of the control antioxidants. There were no significant differences in the ability to reduce iron between extracts obtained by sonication and shaking. Again 20E exhibited weak antioxidant potential (Table 2).

DPPH assay

It is a rapid and easy method for the determination of radical scavenging activity. DPPH is a stable, nitrogen-centered free radical, with absorption maximum at 517 nm. It might be converted to 2, 2-diphenyl-1-pichydrazyl hydrazine by a hydrogen donor (Jayaprakasha et al., 2004), which is observed as a decrease of absorption at 517 nm and decolourization of the solution. The IC_{50} value corresponds to a compound concentration, required to decrease the DPPH absorbance by 50% (Konczak-Islam et al., 2003).

The radical scavenging activity of RCEs towards the DPPH free radical was exhibited by extracts obtained with the use of polar solvents (methanol and water). The methanol extract obtained through sonication turned out to be about 3.5 fold more active than BHT, however about 7 and 3 fold lower than ascorbic acid and α-tocopherol, respectively (Table 2).

In the case of pure 20-hydroxyecdysone in DPPH assay, no antioxidant effect was observed even at the highest concentrations. These observations were contradictory to what was found by several scientists (Osinskaia et al., 1992; Kuzmenko et al., 1997; Miliauskas et al., 2005). Miliauskas and co-workers (Miliauskas et al., 2005) observed that 20E possess mild radical scavenging activity towards DPPH. However, they suggested that the antioxidant effect should be rather ascribed to some other phenolic compounds coeluting with 20E than 20E itself (Miliauskas et al., 2005). The latter explanation seems to be more convincing, since ecdysteoid molecules do not possess structures responsible for antioxidant effect, commonly found in most known antioxidant, eg. o-dihydroxybenzene (catechol) structures in

Table 3. Comparison of the cytototoxic activity of different *R. carthamoides* extracts and 20-hydroxyecdysone.

Analytes	IC_{50} (µg/ml)[a]			
	HeLa	HEK	HL-60	U937
RCE chloroform/sonication	17.7	29.0	12.0	25.6
RCE chloroform/shaking	19.8	17.5	15.0	32.3
RCE methanol/sonication	>50	>50	40.0	>50
RCE methanol/shaking	>50	>50	>50	>50
RCE water/sonication	>50	>50	>50	>50
RCE water/shaking	>50	>50	>50	>50
20E	>50	>50	>50	>50

IC_{50} (µg/ml) = µg of extract in 1 ml of cell culture medium.

flavonoids (Lu and Foo, 2001) or 6-hydroxychroman rings in tocopherols (Prieto et al., 1999).

The results shown above demonstrate the presence of antioxidant agents in *R. carthamoides* extracts, which were able to act as free radical scavengers and prevent lipid peroxidation *in vitro.* RCEs were active both in aqueous environment and emulsion. However the subject demands further investigation, since there are very few reports in this field. Antioxidant potential of *R. carthamoides* total extract was observed previously by Miliauskas et al. (2004), who examined ethyl acetate, acetone and methanol extracts, taking into consideration their radical scavenging activity but not non-radical mechanisms. The present statement, that extracts obtained with the use of polar solvents exhibited stronger radical scavenging activity towards DPPH is in accordance with data shown earlier (Miliauskas et al., 2004).

Moreover it should be emphasised that *R. carthamoides* extracts display rather a complex pattern of antioxidant activity. The most interesting is the case of chloroform extracts, since they do not exhibit even minor activity in the case of DPPH assay and FRAP, showing at the same time high activity in both the other tests. No such phenomenon was observed either for methanol or aqueous extract.

Evaluation of cytotoxic activity of *Rhaponticum carthamoides* extracts

In this study cytotoxic activity of six *R. carthamoides* extracts, as well as the activity of pure 20-hydroxyecdysone was tested against HeLa, HEK, HL-60 and U937 cell lines. The HeLa cell line is a stable, epithelial like cell lines, derived from human uterine cervix carcinoma. HEK is an epithelial like cell line as well, originally isolated from human embryo kidney. HL-60 and U937 are both leukemic cell line. All are easy to maintain and are widely used as model cell lines.

In order to determine the cytotoxic effect of *R. carthamoides* leaves and stems extracts the MTT assay was conducted. This assay allows the determination of the viability of cells (or more precisely their redox activity), using a water soluble tetrazolium salt MTT.

Metabolically active eukaryotic and prokaryotic cells reduce MTT (yellow) to formazan (violet). In the case of *Eukaryota* this process was initially believed to take place only in mitochondria, but recently also non-mitochondrial reduction of MTT was described in the cytosol, endoplasmic reticulum and plasma membranes (Bernas and Dobrucki, 2002). MTT assay results for RCEs and 20E are presented in Table 3.

Cytotoxicity was exhibited only by chloroform extracts. According to Wall et al. (1987) these results may be regarded as significant, since IC_{50} is lower than 20 µg/ml (with the exception of U937). This value was adopted by these researchers based on extensive experience of over a 25-year period in comparing *in vitro* and *in vivo* assays data (Wall et al., 1987). However, according to NCI recommendation an extract/compound has a cytotoxic activity if the IC_{50} value is less than 4 µg/ml (Jackson et al., 2000). According to these data, neither RCEs nor pure 20E can be regarded as having efficient cytotoxic activity.

The cytotoxic activity of *R. carthamoides* leaf extracts was not initially tested, although some attention was put to the influence of the root extract on the human breast cancer cell line MCF-7. The root extract inhibited cell proliferation with the IC_{50} = 28 µg/ml, but no influence of 20E was observed (Hamburger et al., 2006). The latter statement is in accordance with our findings.

Assessment of potential mutagenic activity of *Rhaponticum carthamoides* extracts

Chemicals exhibiting mutagenic properties might be harmful to living organisms, as their interaction with DNA

Table 4. Evaluation of *R. carthamoides* extracts mutagenic activity.

Analytes	Mutagenic activity expressed in percent of neomycin-resistant mutants (percent x 10^5)	
	BB7 (WT strain)	BB7M (*muc*A*muc*B mutant)
RCE chloroform/sonication	82.5 ± 5.0	ND
RCE chloroform/shaking	109.2 ± 9.4	6.3 ± 5.3
RCE methanol/sonication	103.3 ± 24.2	12.5 ± 7.1
RCE methanol/shaking	106.7 ± 11.2	10.0 ± 10.6
RCE water/sonication	78.3 ± 21.4	20.0 ± 6.6
RCE water/shaking	92.5 ± 17.8	17.5 ± 10.9
Sodium azide	132.5 ± 23.9	49.2 ± 14.2
Methanol (vehicle)	110.8 ± 15.5	15.0 ± 4.3
Non-treated sample	111.7 ± 26.4	11.7 ± 3.8

leads to changes in the genetic information. The latter may cause cell death or neoplastic transformation. Crude plant extracts (Elgorashi et al., 2003; Reid et al., 2006) and purified components (eg. furanocoumarins (Schimmer et al., 1991)) were also proved to exert mutagenic activity. Therefore in this study *R. carthamoides* extracts' was evaluated taking this aspect into consideration.

The mutagenic activity of compounds is usually assessed by the Ames test or its modifications, with the use of indicator strains of *Salmonella typhimurium* (eg. TA98 strain) (Schimmer et al., 1991). However in this study the method described by Czyz et al. (2000a) was employed, since it does not require the use of pathogenic *S. typhimurium* and was successfully used for the detection of mutagenic pollutions in the marine environment (Czyz et al., 2003).

V. harveyi, a non-pathogenic, free-living marine bacteria was used as a bioindicator in this method, instead of *S. typhimurium*. *V. harveyi* BB7 (WT) and BB7M strain were susceptible to neomycin, but resistant mutants occurred spontaneously at a constant frequency (ca. 10^{-4} – 10^{-5}). In the presence of mutagenic agents, the frequency of mutations was elevated. The reason for choosing neomycin as a selection agent results from its mode of action. Neomycin is an aminoglicoside antibiotic, which binds to rRNA in the small subunit of the bacterial ribosome and therefore resistance to neomycin is related to several mutations in the rRNA. This feature ensures higher frequency of resistance incidents, than in case of specific substitution mutants (Czyz et al., 2000a).

Several preliminary tests were performed in order to establish the highest extract concentration not influencing bacterial growth (data not shown) and the concentration 50 µg/ml was selected. Two *V. harveyi* strains were used, since they differ in the efficiency of DNA repair systems and consequently in mutation occurrence.

No mutagenic effect was observed in the case of any of the six RCEs tested (Table 4). For WT BB7 the percentage of mutants was comparable with negative controls, but did not differ statistically from the positive control (sodium azide). As far as the BB7M strain is concerned, the occurrence of neomycin-resistant mutants was significantly lower ($p < 0.05$) for RCEs than for sodium azide. This finding supports previous data, concerning low toxicity of *R. carthamoides* extracts (Petkov et al., 1984).

The antioxidant capacity of RCEs, observed in both polar and non-polar environments, with radical and non-radical reaction mechanisms involved, seems to be worth further investigation. It might be crucial to find out whether *R. carthamoides* extracts are capable also of protecting against oxidative stress under *in vivo* conditions.

ACKNOWLEDGMENT

This work was supported by State Committee for Scientific Research. Project Nr PBZ-KBN-092/P05/2003.

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