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**ARTICLES**

**Research Articles**

- Activities Of Guatemalan Medicinal Plants Against Cancer Cell Lines And Selected Microbes: Evidence For Their Conservation** **1040**  
Rex G. Cates, Andrew Thompson, Holly Brabazon, Sidney McDonald, Michael Lawrence, Steven Williams, Pablo Peniallilo, J. Alfonso Fuentes Soria, Luis V. Espinoza, José Vicente Martínez, Dany A. Arbizú, Ernesto Villagran and Fernando Ancheta
- Allelopathic Effect Of Popular Medicinal Plants On Fagopyrum Esculentum (Moench), Papaver Somniferum (L.) And Brassica Napus Var. Oleifera (L.)** **1051**  
Kinga Matysiak, Sylwia Kaczmarek and Roman Kierzek
- Nobiletin, A Polymethoxylated Flavone From Citrus Peels, Induces Differentiation Of Normal Human Epidermal Keratinocytes** **1060**  
Kenji Ohguchi, Munekazu Inuma, Yoshinori Nozawa and Masafumi Ito

## Full Length Research Paper

# Activities of Guatemalan medicinal plants against cancer cell lines and selected microbes: Evidence for their conservation

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Medicinal plants are important components in the primary health care of villagers in Guatemala. However, an area often overlooked is the effect of medicinal plants on oral hygiene. Acetone and methanol extracts from 63 medicinal plant species from 31 families were bioassayed against breast, cervical, skin and tongue cancers, and the following microorganisms: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus mutans*, *Lactobacillus acidophilus* and *Candida albicans*. Half-maximum inhibitory concentrations (IC<sub>50</sub>) and half-maximum cytotoxicity concentrations (CC<sub>50</sub>) were determined against cancerous and non-cancerous cell lines, respectively. Minimum inhibitory concentrations (MIC) were determined against microbes. Based on levels of inhibition by extracts, IC<sub>50</sub> values, CC<sub>50</sub> values, and MIC values, seven species (*Bursera simaruba* Sarg., Burseraceae; *Eriobotrya japonica* (Thumb.) Lindl., Rosaceae; *Litsea guatemalensis* Mez, Lauraceae; *Mirabilis jalapa* L., Nyctaginaceae; *Pithecellobium dulce* (Roxb.) Benth., Fabaceae); *Rubus villosus* Thunb., Rosaceae; *Thevetia peruviana* K. Schum., Apocynaceae) were recommended for additional investigation. With regard to oral hygiene four species (*Achillea millefolium* L., Asteraceae; *Crotalaria longirostrata* Hook. and Arn., Fabaceae; *P. dulce*; *Spondias purpurea* L., Anacardiaceae) may merit further fractionation and testing against oral diseases.

**Key words:** Anticancer, antimicrobial, IC<sub>50</sub>, CC<sub>50</sub>, MIC, oral hygiene, Guatemala.

## INTRODUCTION

Well documented is the use and value of the earth's medicinal resources with regard to primary health care for the human population. For example, Kingston (2011) and Newman and Cragg (2007) suggest that up to 50%

of the drugs now available to treat human diseases are related to natural products. For anticancer, anti-migraine, and other drugs the estimate is well over 50% (Newman and Cragg, 2012; Butler, 2008; McChesney et al., 2007).

However, Newman et al. (2008), Adams and Hawkins (2007), and Chaudhuri (2007) noted that global access to these types of drugs is highly variable. The result is that traditional remedies support the health care of over 65% of the world population (Fabricant and Farnsworth, 2001), and in rural communities the estimate is 75 to 90% (Chivian and Bernstein, 2008; Fowler, 2006), depending on the geographical area.

An additional consideration is that traditional knowledge and the biodiversity that supports that knowledge and the development of new drugs are being lost (Cordell and Colvard, 2012; Strobel et al., 2004). These in combination with the evolution of drug resistance (Lambert et al., 2011) contribute to the increased awareness to conserve these valuable plant resources (Siwach et al., 2013; Kingston, 2011). Another concern regarding the primary health care of people in rural communities worldwide is the lack of information on the role of medicinal plants to improve oral hygiene (Colvard et al., 2006). For example, Kufer et al. (2005) in their study on the use of medicinal plants in the Ch'orto' area in southeastern Guatemala listed about 41 plants that were used to treat gastrointestinal illnesses, 34 species used for fever and pain, 38 for women's remedies, 25 for respiratory illnesses, but only seven for oral health problems. Of these seven, three were used in prevention and all seven were used for toothaches. Rural family members in southeastern Guatemala near Esquipulas who were suffering from toothache or orofacial pain resorted to using nine herbals but no traditional remedies were noted to prevent cavities or other oral cavity diseases (Hunter and Arbona, 1995). Consequently, a need exists to find medicinal plants that have potential to prevent and treat periodontal diseases and other oral health issues.

These concerns are relevant to the health care of villagers in Guatemala and therefore formed the basis for this study. The first objective was to evaluate the *in vitro* growth inhibition of acetone and methanol extracts from 63 plant species against breast, cervical, skin, and tongue cancer cell lines and a non-cancerous line. For those extracts that were inhibitory at 60% or greater  $IC_{50}$  and  $CC_{50}$  values were determined. Secondly, *in vitro* growth inhibition of these extracts against *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, *Lactobacillus acidophilus*, and *Candida albicans* were determined. For those active at 60% or greater minimum inhibitory concentrations (MIC) were obtained. All 63 species are noted in Guatemalan health care pharmacopoeias and about half of these species are used for oral health care. Consequently, activity against *Streptococcus mutans*, *Lactobacillus acidophilus*, *Candida albicans* and the tongue cancer cell line was of

particular interest due to their association with dental plaque, caries, and other oral cavity health issues (Kleinberg, 2002).

## MATERIALS AND METHODS

### Plant collection, tissue preparation, cell lines and microbial cultures

Eighteen species were collected from the Museo Odontológico de Guatemala y Jardín Botánico Maya, Guatemala City, Guatemala, 20 species from Colección y Huerto Productivo de Plantas Medicinales, Facultad de Agronomía, Guatemala City, and 25 from the communities of Olopa and San Juan Ermita in southeastern Guatemala. Aids in identifying species other than vouchers and digital pictures were the *Vademecum Nacional de Plantas Medicinales* (Cáceres, 2009), the guide to medicinal plants by Arevalo and Dieseldorff (2005), and a species list for the Museo Odontológico de Guatemala y Jardín Botánico Maya. Voucher specimens are located in the herbaria at the Centro Universitario de Oriente, Universidad de San Carlos de Guatemala, Chiquimula, Guatemala (CUNORI) and at Brigham Young University (BRY), Provo, UT. Each sample from the 63 species analyzed consisted of tissue (Table 1) collected from three or more individuals that was mixed, then bagged, labelled, and stored at  $-80^{\circ}\text{C}$  (Isotemp Basic, Thermo Electron Corporation, Asheville, NC USA) at BYU. Acetone and methanol extracts derived from five grams of plant tissue were eventually dissolved in double-distilled water at a final concentration of 8 mg/ml. The human cancer cell lines used were breast (ATCC HTB-22, breast mammary gland adenocarcinoma; ATCC, Manassas, VA), HeLa (ATCC CCL-2, cervix epithelial adenocarcinoma; ATCC), skin (ATCC CRL-2095, epithelial malignant melanoma; ATCC), and tongue (ATCC CRL-2095, human epithelial squamous carcinoma; ATCC). Cytotoxicity was determined using a non-cancerous Vero cell line (ATCC CRL-1586, epithelial kidney monkey; ATCC). *Staphylococcus aureus* (ATCC 6538P; Becton Dickinson Laboratories, Cockeysville, MD), *Escherichia coli* (ATCC 11229; ATCC) oral isolates of *Streptococcus mutans* (ATCC 33402, ATCC), *Lactobacillus acidophilus* (ATCC 11975, ATCC) and *Candida albicans* (ATCC 90028, ATCC) were used to determine the antimicrobial activity of acetone and methanol extracts. Methods for culturing cancer cell lines, the non-cancerous cell line, and microbes are described by Cates et al. (2013).

### Sulforhodamine B assay and neutral red (NR) assay

The sulforhodamine B assay used to determine the level of inhibition of extracts against cancer cell lines followed Skehan et al. (1990) and Donaldson et al. (2004) as described by Cates et al. (2013). Inhibition activity against cell lines was determined in triplicate at 200, 100, and 50  $\mu\text{g/ml}$  of extract. Results in Table 2 are reported only for the 200  $\mu\text{g/ml}$  concentration. The NR assay followed Putnam et al. (2002) and was used on all extracts that showed 60% or greater inhibition in the sulforhodamine assay. Serial dilutions of 200, 100, 50, 25, 12.5 and 6.25  $\mu\text{g/ml}$  of each plant extract were run in triplicate against each cell line (Cates et al., 2013). Additional concentrations of extract were included in the NR assay so that more data would be available for accurate calculation of half-maximum inhibitory concentrations

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**Table 1.** Scientific names, common names, tissue collected, and use of medicinal plants.

Scientific name	Family	Common name	Tissue extracted	Medicinal use
<i>Acacia farnesiana</i> (L.) Willd.	Leguminosae	Subin	Leaves	Vaginal bleeding, fertility, after childbirth, cold*
<i>Acalypha guatemalensis</i> Pax & K. Hoffm.	Euphorbiaceae	Hierba de cancer	Leaves	Gum disease, tooth ache, cancer
<i>Achillea millefolium</i> L.	Asteraceae	Milenrama	Aerial portion	Fever, colds, dysentery
<i>Allium sativum</i> L.	Liliaceae	Ajo	Bulb	Digestion disorders, respiratory diseases
<i>Anethum graveolens</i> L.	Apiaceae	Hinojo	Leaves	Diarrhea, after birth antiseptic, stomach pain
<i>Anthemis oppositifolia</i> Lam.#	Asteraceae	Ixmaramac	Leaves	Anesthetic
<i>Arnica montana</i> L.†	Asteraceae	Arnica	Aerial portion	
<i>Asclepias curassavica</i> L.	Apocynaceae	Cuajatinga	Leaves	Fever
<i>Baccharis trinervis</i> Pers.	Asteraceae	Corrimiento	Leaves	Analgelsic
<i>Bourreria huanita</i> (Lex.) Hemsl.	Boraginaceae	Esquisuchil	Leaves	Fever, cold
<i>Brosimum alicastrum</i> Sw.	Moraceae	Ramon (Ujuxte)	Green fruit	Cough, sore throat
<i>Brugmansia candida</i> Pers.	Solanaceae	Florifundia	Leaves	Tooth ache pain, sleep agent
<i>Bursera simaruba</i> (L.) Sarg.	Burseraceae	Palo de jote	Leaves	Wounds, insect bites, stings
<i>Casimiroa edulis</i> La Llave & Lex.	Rutaceae	Matasano	Roots	Birthing accelerant
<i>Cedrela odorata</i> L.	Meliaceae	Cedro	Bark (inner)	Tooth pain, birthing accelerant
<i>Cinnamomum zeylanicum</i> Blume	Lauraceae	Canela	Leaves	Fever, headache, cold, diarrhea
<i>Citrus sinensis</i> (L.) Osbeck	Rutaceae	Naranja	Leaves	Anxiety, depression
<i>Coffea arabica</i> L.	Rubiaceae	Café	Leaves	Dizziness
<i>Costus pictus</i> D. Don†	Costaceae	Cana de cristo	Leaves	
<i>Crotalaria longirostrata</i> Hook & Arn.	Fabaceae	Chipilin	Leaves	Sedative, anemia, insomnia
<i>Cupressus lusitanica</i> Mill.	Cupressaceae	Cipres	Needles	Cough
<i>Equisetum arvense</i> L.	Equisetaceae	Oreja de coche	Aerial portion	Gripe
<i>Eriobotrya japonica</i> (Thumb.) Lindl.	Rosaceae	Nispero	Green fruit	Tooth pain, gum inflammation
<i>Euphorbia lancifolia</i> Schldt.	Euphorbiaceae	Ixbut	Leaves	Lactation stimulate, impotence, cold
<i>Fleischmannia pycnocephala</i> (Less.) R. M. King and H. Rob.	Asteraceae	Violeta‡	Aerial portion	Respiratory problems
<i>Hibiscus sabdariffa</i> L.	Malvaceae	Rosa de Jamaica	Leaves	Intestinal distress, chicken pox
<i>Ixora coccinea</i> L.	Rubiaceae	Coralillo	Leaves	Muscle relaxant
<i>Jatropha curcas</i> L.	Euphorbiaceae	Pinon	Leaves	Kidney and intestinal problems, heartburn, inflamed gums
<i>Latana camara</i> L.	Verbenaceae	Cinco negritos	Leaves	Female hemorrhaging, discharge
<i>Lippia dulcis</i> Trevir.	Verbenaceae	Orosus	Aerial portion	Bronchitis
<i>Lippia graveolens</i> Kunth	Verbenaceae	Oregano	Aerial portion	Pain from tooth ache, spice
<i>Litsea guatemalensis</i> Mez	Lauraceae	Laurel	Leaves	Gastrointestinal problems, colic, swelling
<i>Mirabilis jalapa</i> L.	Nyctaginaceae	Flor de maravilla	Aerial portion	Cold, influenza, diarrhea
<i>Murraya paniculata</i> (L.) Jack	Rutaceae	Limonaria	Leaves	Relieve tooth ache pain
<i>Nicotiana tabacum</i> L.	Solanaceae	Tabaco	Leaves	Tooth ache
<i>Ocimum basilicum</i> L.	Lamiaceae	Albahaca morada	Aerial portion	Gastrointestinal spasm, migraine headaches
<i>Ocimum micranthum</i> Willd.	Lamiaceae	Albahaca del monte	Aerial portion	Stomach ache
<i>Origanum vulgare</i> L.	Lamiaceae	Oregano de Castillo	Aerial portion	Menstruation
<i>Passiflora ligularis</i> Juss.	Heliconiaceae	Granadilla	Leaves	Anxiety, tooth ache pain
<i>Persea americana</i> Mill	Lauraceae	Aguacate	Leaves	Tooth ache, rheumatism, cough
<i>Petiveria alliacea</i> L.	Phytolaccaceae	Apacin	Leaves	Fever, nasal congestion, gastritis, diarrhea
<i>Pimenta dioica</i> (L.) Merr.	Myrtaceae	Peinata	Leaves	Anesthetic, stomach pain
<i>Piper auritum</i> Kunth	Piperaceae	Santa Maria	Leaves	Cancer
<i>Pithecellobium dulce</i> (Roxb.) Benth.	Fabaceae	Shaguay	Bark	Kidney stones
<i>Priva lappulacea</i> (L.) Pers.	Verbenaceae	Mozotillo	Leaves	Kidney disease
<i>Prunus persica</i> (L.) Batsch	Rosaceae	Durango	Leaves	Cold, cough, eating
<i>Punica granatum</i> L.	Lythraceae	Granado	Leaves	Tooth ache, diarrhea
<i>Rauvolfia tetraphylla</i> L.	Apocynaceae	Chalchupa	Leaves	Hypertension
<i>Rhus terebinthifolia</i> Schlecht & Cham	Anacardiaceae	Sal de venado	Leaves	Tooth ache pain, gum disease
<i>Rosmarinus officinalis</i> L.	Lamiaceae	Romero	Aerial portion	Colic, bronchitis, anemia
<i>Rubus villosus</i> Lasch.	Rosaceae	Sarzamora	Leaves	Cold, cough, influenza, diarrhea, parasites
<i>Senna occidentalis</i> L.	Fabaceae	Frijolillo	Leaves	Dental disease
<i>Solanum torvum</i> Sw.	Solanaceae	Chichita	Leaves	Bronchitis, cold, diarrhea
<i>Solanum umbellatum</i> Miller#	Solanaceae	Tabaquillo	Leaves	Cleaning powder for teeth, tooth ache
<i>Spondias purpurea</i> L.	Anacardiaceae	Jocote	Leaves	Astringent, diarrhea, dysentery



Table 1 cont'd

<i>Stigmaphyllon ellipticum</i> A. Juss.	Malpighiaceae	Contra hierba	Leaves	Snake bites, tooth ache
<i>Tagetes filifolia</i> Lag.	Asteraceae	Anis de monte	Leaves	Stomach ache, diarrhea <sup>#</sup>
<i>Tagetes lucida</i> Cav.	Asteraceae	Pericon	Leaves	Abdominal and menstrual pain
<i>Taraxacum officinale</i> F. H. Wigg.	Asteraceae	Amargon	Leaves	Hepatic and urinary disorders
<i>Thevetia peruviana</i> Merr.	Apocynaceae	Quiebra la muela	Leaves	Paste applied to cavity for tooth removal
<i>Thymus vulgaris</i> L.	Lamiaceae	Tomillo	Aerial portion	Respiratory infections, bronchitis, cough
<i>Tridax procumbens</i> L.	Asteraceae	Hierba del toro	Aerial portion	Hemorrhage
<i>Vetiveria zizanioides</i> (L.) Nash <sup>†</sup>	Poaceae	Vetiver grass (Valeriana) <sup>‡</sup>	Leaves	

<sup>†</sup>Information from Kufer et al. (2005).

<sup>#</sup>*Anthemis oppositifolia* and *Solanum umbellatum* were not analyzed for activity against microbes due to lack of tissue.

<sup>†</sup>Medicinal use not clearly defined at time of collection.

<sup>‡</sup>Local villagers referred to *V. zizanioides* as Valeriana and *F. pycnocephala* as violet.

(IC<sub>50</sub>) and half-maximum cytotoxicity concentrations (CC<sub>50</sub>). The IC<sub>50</sub> and CC<sub>50</sub> values were obtained using dosage response curves.

#### Microbial inhibition assay and minimum inhibitory concentrations (MIC)

To determine which extracts exhibited inhibition against the pathogens a microwell dilution bioassay was performed using 1000, 500, and 250 µg/ml of extract following Shrestha and St. Clair (2013). Each extract was tested in triplicate and only percent inhibition at the 1000 µg/ml concentration was reported (Table 4). For plant extracts that were inhibitory at 60% or greater (Table 4) MICs were determined using a microwell dilution bioassay. Concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml were tested in triplicate against the microbes. The MIC was defined as the lowest concentration of extract at which no reduction of *p*-iodonitro-tetrazolium violet dye (Sigma-Aldrich) was observed. MICs were not calculated for *S. mutans* and *L. acidophilus* due to irregular growth and clumping. Details of these two assays are found in Cates et al. (2013).

#### Data analysis

Data were coded by species and fraction and statistical significance ( $P \leq 0.001$ ) between control vs. inhibition values were determined by ANOVA (R Core Team, 2013). Results from the 200 µg/ml concentration used against cancer cell lines and the 1000 µg/ml concentration used against the microbes are the only results reported (Tables 2 and 4). This is because these concentrations yielded the maximum number of active plant species. Consequently, any extract showing greater than 60% inhibition for the acetone or methanol extracts at the 200 µg/ml level for any cancer cell line, and at the 1000 µg/ml for any microbial species, was considered active and worthy of neutral red or MIC analysis. An additional criterion was that if the inhibition level of a cancer cell line was two to three times that of the Vero line then those extracts were considered active.

## RESULTS

### Sulphorhodamine inhibition and cytotoxicity to Vero cells

Eight (12.7%) of the 63 species analyzed showed activity

against one or more of the cancer cell lines (Table 2). The acetone extracts of *Persea americana* Mill. (Lauraceae) and *Pithecellobium dulce* (Roxb.) Benth. (Fabaceae) were active against breast cancer cells (97% and 73% inhibition, respectively). The methanol extract (96%) of *Bursera simaruba* (L.) Sarg. (Burseraceae) and the acetone and methanol extracts (70 and 60%, respectively) of *Litsea guatemalensis* Mez (Lauraceae) were also active against this cell line. The acetone extract (94%) from *P. americana* and the methanol extract (75%) of *Cedrela odorata* L. (Meliaceae) were active against the HeLa line (Table 2). Acetone and methanol (68 and 69%, respectively) extracts from *Solanum umbellatum* Miller (Solanaceae) and *Thevetia peruviana* Merr. (Apocynaceae) (60 and 68%, respectively) also were active against this line. *Crotolaria longirostrata* Hook. and Arn. (Fabaceae) produced an acetone extract that was active against skin and tongue cell lines (62% and 61% inhibition, respectively), and the methanol extract (62%) of *T. peruviana* was active against the skin cancer cell line (Table 2). However, the acetone extracts from *C. longirostrata*, *P. dulce* and the acetone and methanol extracts from *T. peruviana* showed cytotoxic effects against the non-cancerous Vero cell line.

### Neutral red (NR) assay for inhibition and cytotoxicity

The methanol extract from *B. simaruba* and the acetone extract from *T. peruviana* were highly inhibitory at low concentrations (IC<sub>50</sub> = 75 µg/ml and 30 µg/ml, respectively) against the breast and HeLa cancer cell lines, respectively (Table 3). They also yielded low inhibition at high concentrations against Vero cells (CC<sub>50</sub> > 800 µg/ml and 663 µg/ml, respectively). The acetone extract from *L. guatemalensis*, and to some extent the acetone extract from *P. americana*, showed moderate activity against the breast and HeLa lines (IC<sub>50</sub> = 226 µg/ml and 387 µg/ml, respectively), and low inhibition at high concentrations against the Vero line (CC<sub>50</sub> > 800 µg/ml). The other species showed high IC<sub>50</sub> and/or low



Table 2 cont'd

<i>Rosmarinus officinalis</i>	2±1	3±1	0	0	19±6	0	0	0	-	-
<i>Rubus villosus</i>	0	0	0	9±4	0	0	0	0	5±1	-
<i>Senna occidentalis</i>	0	0	0	0	11±7	0	6±3	0	-	-
<i>Solanum torvum</i>	9±3	0	0	0	0	0	0	0	0	-
<i>Solanum umbellatum</i>	0	0	68±3	69±1	4±1	0	0	0	18±4	0
<i>Spondias purpurea</i>	18±1	0	0	0	0	0	0	0	-	-
<i>Stigmaphyllon ellipticum</i>	0	0	0	0	0	0	0	5±3	0	0
<i>Tagetes filifolia</i>	0	0	12±1	0	0	0	0	0	-	-
<i>Tagetes lucida</i>	0	0	0	0	0	3±1	0	0	5±1	0
<i>Taraxacum officinale</i>	0	0	0	0	0	0	6±3	0	-	-
<i>Thevetia peruviana</i>	30±4	34±10	60±10	68±1	51±5	62±7	39±7	42±12	62±4	59±2
<i>Thymus vulgaris</i>	0	0	6±3	0	0	0	0	4±1	-	-
<i>Tridax procumbens</i>	0	0	0	0	0	0	0	0	-	-
<i>Vetiveria zizanioides</i>	0	0	0	0	0	0	0	0	0	11±5

\*All comparisons between values at 60% or greater inhibition and their controls were significantly different at  $P \leq 0.001$ .

**Table 3.** Half-maximum inhibitory concentration (IC<sub>50</sub>) for cancer lines and half-maximum cytotoxicity concentration (CC<sub>50</sub>) for the Vero cell line.

Cancer cell line/plant species	IC <sub>50</sub> (μ/ml)		CC <sub>50</sub> (μ/ml)	
	A	M	A	M
<b>Breast</b>				
<i>Thevetia peruviana</i>	487	592	663	<6
<i>Bursera simaruba</i>	-	75	-	>800
<i>Pithecellobium dulce</i>	734	-	267	-
<i>Ocimum micranthum</i>	>800	-	>800	-
<i>Litsea guatemalensis</i>	226	-	>800	-
<b>HeLa</b>				
<i>Thevetia peruviana</i>	30	85	663	<6
<i>Persea americana</i>	387	667	>800	>800
<i>Solanum umbellatum</i>	365	315	278	354
<b>Skin</b>				
<i>Thevetia peruviana</i>	800	25	663	<6
<i>Crotalaria longirostrata</i>	168	-	136	-
<b>Tongue</b>				
<i>Thevetia peruviana</i>	>800	>800	663	<6
<i>Crotalaria longirostrata</i>	492	-	136	-

CC<sub>50</sub> values.

### Microbial inhibition

Thirteen (21.3%) of the 61 species tested showed growth inhibition at 60% or greater against one or more microbes (Table 4). Acetone extracts from *Eriobotrya japonica*

(Thumb.) Lindl. (Rosaceae), *Mirabilis jalapa* L. (Nyctaginaceae), *P. americana*, *Pimenta dioica* (L.) Merr. (Myrtaceae), *Priva lappulacea* (L.) Pers. (Verbenaceae), and *Rubus villosus* Lasch. (Rosaceae) were active against *S. aureus*. Methanol extracts from *B. simaruba*, *C. odorata*, and *Murraya paniculata* (L.) Jack (Myrtaceae) were also active against *S. aureus*, as were the acetone and methanol extracts from *P. dulce* (Table 4). Methanol

**Table 4.** The effect of acetone and methanol extracts on microbes.

Genus/Species	% Inhibition (1000 µg/ml)*									
	<i>S. aureus</i>		<i>S. mutans</i>		<i>E. coli</i>		<i>L. acidophilus</i>		<i>C. albicans</i>	
	A	M	A	M	A	M	A	M	A	M
<i>Acacia farnesiana</i>	5±2	38±2	8±4	57±1	16±6	32±3	0	0	0	0
<i>Acalypha guatemalensis</i>	22±1	0	0	0	10±5	0	0	0	0	0
<i>Achillea millefolium</i>	0	0	9±4	51±5	8±1	95±1	20±5	98±1	0	8±3
<i>Allium sativum</i>	0	0	0	24±1	4±1	0	9±5	0	0	0
<i>Anethum graveolens</i>	5±2	0	0	7±3	0	10±4	-	-	0	0
<i>Arnica montana</i>	22±2	0	0	26±1	0	19±3	0	21±2	0	0
<i>Asclepias curassavica</i>	0	16±5	12±3	19±3	0	11±1	35±5	0	0	27±2
<i>Baccharis trinervis</i>	0	33±4	0	0	12±2	0	0	0	5±1	0
<i>Bouyeria huanita</i>	0	0	0	0	0	0	0	0	0	0
<i>Brosimum alicastrum</i>	0	12±3	10±4	0	6±2	0	0	0	0	0
<i>Brugmansia candida</i>	0	0	11±3	4±1	38±1	0	0	16±7	0	0
<i>Bursera simaruba</i>	0	68±1	23±2	36±1	14±3	56±2	0	0	0	0
<i>Casimiroa edulis</i>	0	58±1	12±2	37±1	12±1	5±1	0	27±6	0	0
<i>Cedrela odorata</i>	33±4	84±3	0	0	0	-	-	-	0	0
<i>Cinnamomum zeylanicum</i>	0	0	0	52±1	0	40±2	-	-	0	0
<i>Citrus sinensis</i>	22±4	35±9	-	0	0	0	12±5	24±5	-	0
<i>Coffea arabica</i>	0	0	0	0	29±5	9±4	22±1	19±2	0	0
<i>Costus pictus</i>	0	0	0	0	13±7	0	17±1	39±4	0	0
<i>Crotalaria longirostrata</i>	0	0	0	0	22±1	0	65±3	30±1	16±4	10±7
<i>Cupressus lusitanica</i>	0	0	0	0	0	0	0	0	7±1	0
<i>Equisetum arvense</i>	0	0	0	0	0	0	0	0	0	0
<i>Eriobotrya japonica</i>	62±3	15±5	-	31±4	89±1	15±6	0	0	0	0
<i>Euphorbia lancifolia</i>	0	0	0	0	0	0	0	0	0	0
<i>Fleischmanni pycnocephala</i>	0	0	0	0	0	0	0	0	0	0
<i>Hibiscus sabdariffa</i>	0	0	15±7	19±3	21±2	0	27±2	29±1	0	21±1
<i>Ixora coccinea</i>	0	0	0	0	0	0	20±8	0	0	0
<i>Jatropha curcas</i>	0	0	20±5	16±1	10±6	0	0	0	0	0
<i>Lantana camara</i>	0	0	0	0	18±5	0	0	6±2	0	0
<i>Lippia dulcis</i>	0	0	14±2	15±1	0	0	22±2	21±4	0	20±7
<i>Lippia graveolens</i>	0	0	0	0	0	0	18±1	0	0	0
<i>Litsea guatemalensis</i>	0	0	41±5	17±4	0	0	-	-	0	0
<i>Mirabilis jalapa</i>	60±6	48±5	36±3	0	17±4	0	0	0	0	9±3
<i>Murraya paniculata</i>	0	98±1	9±3	0	15±3	0	0	0	0	0
<i>Nicotiana tabacum</i>	0	0	0	0	0	0	0	0	0	0
<i>Ocimum basilicum</i>	0	0	15±3	12±3	16±6	26±1	34±2	10	0	0
<i>Ocimum micranthum</i>	0	0	32±3	0	0	0	0	0	0	0
<i>Origanum vulgare</i>	0	0	18±4	1	0	0	0	0	0	29±3

Table 4. Cont'd.

<i>Passiflora ligularis</i>	0	0	0	0	0	0	0	0	0	0
<i>Persea americana</i>	64±5	29±4	41±3	26±2	0	0	15±3	0	0	0
<i>Petiveria alliacea</i>	0	0	0	0	0	0	0	6	0	0
<i>Pimenta dioica</i>	60±3	19±9	18±6	0	43±1	29±5	0	0	0	0
<i>Piper auritum</i>	0	7±1	23±4	33±6	23±2	0	0	0	0	0
<i>Pithecellobium dulce</i>	90±3	85±4	0	61±7	90±1	89±2	-	-	0	0
<i>Priva lappulacea</i>	83±1	0	28±9	13±2	0	0	59±1	0	0	0
<i>Prunus persica</i>	0	0	-	0	14±1	12±3	10±2	15±1	0	0
<i>Punica granatum</i>	44±3	29±7	28±2	23±6	12±4	0	0	0	0	0
<i>Rauvolfia tetraphylla</i>	0	0	0	0	0	0	0	0	0	0
<i>Rhus terebinthifolia</i>	28±9	17±2	24±7	22±6	36±2	18±3	0	0	0	0
<i>Rosmarinus officinalis</i>	23±2	0	0	0	0	17±6	0	0	-	0
<i>Rubus villosus</i>	78±2	16±1	0	0	45±6	38±1	0	0	0	0
<i>Senna occidentalis</i>	0	0	0	18±2	0	0	0	23±6	0	0
<i>Solanum torvum</i>	0	0	0	31±3	0	0	0	0	0	0
<i>Spondias purpurea</i>	0	45±4	-	98±3**	0	0	35±5	13±4	0	0
<i>Stigmaphyllon ellipticum</i>	0	0	39±1	20±7	0	0	18±2	0	0	0
<i>Tagetes filifolia</i>	0	25±1	0	0	0	0	0	0	0	0
<i>Tagetes lucida</i>	0	0	0	0	0	0	17±3	0	0	0
<i>Taraxacum officinale</i>	28±4	0	0	0	0	0	0	19±5	0	0
<i>Thevetia peruviana</i>	0	0	16±5	0	24±2	0	0	0	0	0
<i>Thymus vulgaris</i>	0	0	0	13±3	0	0	22±3	25±7	0	31±6
<i>Tridax procumbens</i>	0	0	0	0	0	0	0	0	0	0
<i>Vetiveria zizanioides</i>	0	0	0	0	0	0	0	0	0	0

\*All comparisons between values at 60% or greater inhibition and their controls were significantly different at  $P \leq 0.001$  except for *S. purpurea*\*\* which was significantly different at  $P \leq 0.03$ .

extracts from *P. dulce* and *Spondias purpurea* L. (Anacardiaceae) were inhibitory to the growth of *S. mutans*; no acetone extract was active against *S. mutans* (Table 4). The acetone extract from *E. japonica*, the methanol extract from *Achillea millefolium* L. (Asteraceae), and the acetone and methanol extracts from *P. dulce* were active against *E. coli*. The methanol extract of *A. millefolium* and the acetone extract of *C. longirostrata* were the only extracts active against *L. acidophilus*. No extracts were active against

*C. albicans* (Table 4).

#### Minimum inhibitory concentrations (MICs)

The acetone extracts of *M. jalapa*, *P. dioica*, and *R. villosus* yielded MIC values of 250 µg/ml against *S. aureus* (Table 5). The methanol extract of *B. simaruba* produced an MIC of >1000 µg/ml against *S. aureus*, and a MIC of 500 µg/ml against *E. coli* (Table 5) even though it was not inhibitory

to *E. coli* in the inhibition assay (Table 4). Extracts from *E. japonica* and *P. dulce* yielded extracts with a MIC of 1000 µg/ml; all other extracts yielded MIC values >1000 µg/ml and were not considered inhibitory.

#### DISCUSSION

Our study along with Kufer et al. (2005) and Comerford (1996) note a wide variety of uses for

**Table 5.** Minimum inhibitory concentrations (MIC) for Guatemalan medicinal plants that showed greater than 60% inhibition against microbes.

Plant species (Extract)*	MIC ( $\mu\text{g/ml}$ )	
	<i>S. aureus</i>	<i>E. coli</i>
<i>Achillea millefolium</i> (M)	-	>1000
<i>Bursera simaruba</i> (M)	>1000	500
<i>Cedrela odorata</i> (M)	>1000	
<i>Eriobotrya japonica</i> (A)	>1000	1000
<i>Lantana camara</i> (M)	>1000	
<i>Priva lappulacea</i> (A)	>1000	-
<i>Mirabilis jalapa</i> (A)	250	-
<i>Murraya paniculata</i> (A)	>1000	>1000
<i>Persea americana</i> (A)	>1000	-
<i>Pimenta dioica</i> (A)	250	-
<i>Pithecellobium dulce</i> (A,M)	>1000	1000
<i>Rubus villosus</i> (A)	250	-
<i>Spondias purpurea</i> (A)	>1000	-

\*A=acetone extract; M=methanol extract; blank space indicates no inhibition per Table 4.

the medicinal plants selected for this study (Table 1). This suggests that these resources are valuable to rural Guatemalans and need to be conserved. Overall, 16 (25.4%) of 63 species were inhibitory to one or more cancer cell lines and/or one or more microbes at the 60% or greater level. Eight species were inhibitory to one or more cancer cell lines and eight were inhibitory to one or more microbes (Tables 2 and 4). Of those active against cancer cells, extracts from *B. simaruba* and *L. guatemalensis* demonstrated significant inhibition at low concentrations ( $\text{IC}_{50}$  75 and 226  $\mu\text{g/ml}$ , respectively) against the breast cell line and showed low inhibition at high concentrations ( $\text{CC}_{50}$  >800  $\mu\text{g/ml}$ ) against the non-cancerous Vero cells (Table 3). The acetone extract from *T. peruviana* also demonstrated significant activity against the HeLa cell line ( $\text{IC}_{50}$  30  $\mu\text{g/ml}$  vs  $\text{CC}_{50}$  663  $\mu\text{g/ml}$ ). *P. americana* showed some activity against the HeLa line and with further fractionation this species might prove effective against this line. For the eight species that were active against one or more microbes three (*M. jalapa*, *P. dioica* and *R. villosus*) registered a MIC of 250  $\mu\text{g/ml}$  against *S. aureus*. *B. simaruba* was inhibitory to *S. aureus* (Table 4) but the MIC for the methanol extract was >1000  $\mu\text{g/ml}$  (Table 5). Interestingly the methanol extract from *B. simaruba* was almost significant at 54% inhibition to *E. coli* (Table 4) and that level of inhibition was reflected in a moderately inhibitory MIC of 500  $\mu\text{g/ml}$  against *E. coli* (Table 5). Extracts from *C. odorata*, *C. longirostrata*, *B. simaruba*, *P. americana*, and *P. dulce* were inhibitory to both cancer cell lines and microbes (Table 2 and 4). However, extracts from these five species did not demonstrate significant  $\text{IC}_{50}$ ,  $\text{CC}_{50}$ , or MIC values (Tables 3 and 5). The stated uses of these species

by villagers did not include cancer and microbial diseases (Table 1) so likely the ethnomedical use will not change. Even so, because these species were active against cancer cells and microbes further study of these species may yield promising results.

One focus was to identify medicinal plant species that might be used to improve oral hygiene. Specific emphasis was on plant species demonstrating activity against *S. mutans* and *L. acidophilus* both of which may contribute to cavity formation, and those active against the tongue cancer cell line. *S. purpurea* and *P. dulce* demonstrated significant inhibitory activity against *S. mutans* (Table 4). *C. longirostrata* was inhibitory to the tongue cancer cell line (Table 2), and this species along with *A. millefolium* (and *P. lappulacea* was almost inhibitory at 59% inhibition) were active against *L. acidophilus*. These species merit further investigation as to their efficacy to prevent or treat diseases of the oral cavity.

Several species reported in this study have been reported elsewhere to have activity against human diseases. For example, Johnson (1999) refers to extracts from *B. simaruba* and *P. americana* as being used to treat stomach cancer and tumors, respectively, and in our study these species were active against breast and cervical cancer cells, respectively. Additionally, *S. umbellatum* is an important medicinal plant in some cultures but was not reported to have activity against cancer cell lines (Johnson, 1999).

However, in our study this species was active against cervical cancer cells. In summary, data from this study yielded 11 significantly active species and Cates et al. (2013) noted seven additional active species. Miller

(2014) found 11 other Guatemalan species that produced essential oils which were highly active against the same set of microbes used in this study which brings the total to 29 active medicinal plant species. Future work is needed to determine the pharmacological activity and cytotoxicity of active components. For example, *T. peruviana* was active against the HeLa cell line but is well known for its cytotoxicity (Bandara et al., 2010). Additional studies of the active species might include characterizing the active compounds, and *in vitro* and *in vivo* investigations of their cytotoxicity, mechanism(s) of action, and ultimately their efficacy in preventing and treating diseases.

## Conclusion

Sixteen species of medicinal plants were found to be inhibitory to one or more cancer cell lines and/or microbes. Based on cytotoxicity to the Vero cell line, high IC<sub>50</sub> values and low CC<sub>50</sub> values, and high MIC values several of these species may not merit further study. However, seven species (*B. simaruba*, *E. japonica*, *L. guatemalensis*, *M. jalapa*, *P. dioica*, *R. villosus*, *T. peruviana*) merit additional investigation based on their inhibition, IC<sub>50</sub>/CC<sub>50</sub> values, and MIC values. With regard to oral hygiene four species (*A. millefolium*, *C. longirostrata*, *P. dulce*, *S. purpurea*) merit further fractionation and testing against various oral diseases.

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## Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

## Allelopathic effect of popular medicinal plants on *Fagopyrum esculentum* (Moench), *Papaver somniferum* (L.) and *Brassica napus var. oleifera* (L.)

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Glasshouse experiments were carried out to assess the effect of popular medicinal herbs dry matter on germination and early growth of common buckwheat (*Fagopyrum esculentum*), opium poppy (*Papaver somniferum*) and two cultivars of oilseed rape (*Brassica napus var. oleifera*). Depending on species of the herb, leaves, inflorescences, fruits and whole plants were used. The greatest stimulatory effect on seedling emergence of common buckwheat was exhibited by *Urtica sp.* while seedling emergence of opium poppy was most affected by *Hypericum sp.* Seedling emergence of common buckwheat was reduced by the use of *Matricaria sp.* inflorescence, while *Euphrasia sp.* herb reduced emergence of opium poppy. *Urtica sp.* leaves caused a significant increase in fresh matter of buckwheat as well as opium poppy. The greatest reduction in fresh matter of buckwheat was noted as a result of using *Euphrasia sp.* herb, while in the case of opium poppy, fresh matter was most reduced by using *Tilia sp.* inflorescence. *Mentha sp.* exhibited a strong stimulatory effect on seedling emergence of oilseed rape cultivar Californium, while *Achillea sp.* had an inhibitory influence. All the examined medicinal plants inhibited seedling emergence of semi-dwarf hybrid oilseed rape cv. Maximus. Inhibitory or a stimulatory effect on germination of winter oilseed rape seeds was not always correlated with a reduction or an increase in plant fresh matter.

**Key words:** Early growth, fresh mass, germination, herbs.

### INTRODUCTION

At present, there is an all-world tendency to decrease the amount of chemicals used in agricultural production by introduction of up-to-date biological and ecological methods. One possible solution is integrated plant protection, using among other things, the phenomenon of allelopathy (Aziz and Fuji, 2006; Hussain et al., 2007). A stimulatory or inhibitory effect of one species on another

is a common phenomenon in the plant world. However, it is still little known. The term allelopathy was created by Austrian physiologist Molisch and initially stood for biochemical interactions between higher plants and microorganisms. Only later researches on allelopathy were focused on isolation of a substance (allelopathin), its chemical identification and influence on other plants.

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Currently, allelopathy is considered as not only a phenomenon but also as a new field of chemical ecology (Jeziarska-Domaradzka and Kuźniewski, 2007). Depending on the secreting organism (so-called donor) and a receiving organism (so-called acceptor), allelopathic compounds is divided into four groups: kolines, phytoncides, marasmins and antibiotics (Wójcik-Wojtkowiak et al., 1998). Active substances are commonly called allelopathic compounds or substances, allelopathics or allelopathins (Aldrich, 1997). According to the International Allelopathy Society, allelopathy is “each process associated with secondary metabolites produced by plants, algae, bacteria and fungi which affect growth and development of agricultural and biological systems” (Anonymus, 1996). So far, world literature data have confirmed the allelopathic influence of some plants on cultivated plants. They include such species as: *Comellina alyassum*, exhibiting a stimulatory effect on flax, *Setaria faberi*, inhibiting corn growth, *Rumex crispus*, promoting germination and growth of corn and sorghum, *Pteridium esculentum*, causing intensive elongation growth of alfalfa, and *Conyza canadiensis*, stimulating corn germination (Hussain et al., 2007). As it is stated by Gniazdowska et al. (2004), allelopathic compounds have a diverse effect. However, their most common effect described in literature is influence on the process of seed germination as well as on growth and development of seedlings. Allelopathins have been found in all organs, both vegetative and generative. However, it is stated that compounds derived from vegetative organs are of relatively the greatest importance, and they are the most abundant in leaves (Rice, 1984; Einhellig, 1995). In literature, there are many data on the use of the phenomenon of allelopathy in controlling weed infestation (Bhowmik and Inderjit, 2003; Singh et al., 2003; Vyvyan, 2002). In the present study, we describe the influence of some popular medicinal plants on early growth and amount of generated above-ground plant matter of common buckwheat opium poppy and two cultivars of oilseed rape.

## MATERIALS AND METHODS

Glasshouse experiments were conducted to assess the influence of distribution of dry matter of various herb species in soil on germination and growth of common buckwheat (*Fagopyrum esculentum* Moench. cv. Kora), opium poppy (*Papaver somniferum* L. cv. Mieszko) and winter oilseed rape (*Brassica napus* var. *oleifera* cv. Californium) and semi-dwarf hybrid cultivar Maximus. The experiments used inflorescences of marigold (*Calendula* sp.), chamomile (*Matricaria* sp.), hawthorn (*Crataegus* sp.) and linden (*Tilia* sp.), leaves of mint (*Mentha* sp.), nettle (*Urtica* sp.) and sage (*Salvia* sp.), herb of yarrow (*Achillea* sp.), pansy (*Viola* sp.), St John's wort (*Hypericum* sp.), euphrasia (*Euphrasia* sp.), horsetail (*Equisetum* sp.), and fennel fruit (*Foeniculi* sp.). The analyzed medicinal plants are representatives of 10 botanical families, and dry matter of the whole plants or their individual parts used in the experiment was 5 g. Detailed information on the used species of

medicinal plants is presented in Table 1. Four separate experiments on common buckwheat, opium poppy and two cultivars of oilseed rape were established in two series, with four replications. Dry matter of herbaceous plants was evenly distributed in the superficial soil layer in pots with a diameter of 10 cm. Thirty nutlets of common buckwheat, seeds of opium poppy and each cultivar of oilseed rape were placed in the prepared soil. Two weeks after sowing, seedling emergence of the cultivated plants was calculated, expressed as plants per pot. Seedling emergence expressed as a percentage was determined, adopting 30 nutlets of buckwheat and poppy and oilseed rape seeds, sown in the beginning of the experiment, as 100%. Results present also a percentage increase or a reduction in the number of germinated cultivated plants in the objects, where the medicinal plants were used in comparison with the number of plants noted in control. After the assessment, density of plants was reduced to a number of 10 plants in each pot. The number was maintained until the end of the experiment. After another three weeks, analysis of fresh matter of 10 plants was conducted. On the basis of the obtained results regarding fresh matter, its percentage increase or reduction was calculated in comparison with control. An increase or a reduction in fresh matter of the collected cultivated plants was determined, considering matter of buckwheat poppy and oilseed rape plants obtained from control as 100%. The results were statistically calculated with the use of analysis of variance at significance level  $LSD_{\alpha=0.05}$ . The statistical analysis was performed in FR – ANALWAR – 4.3.

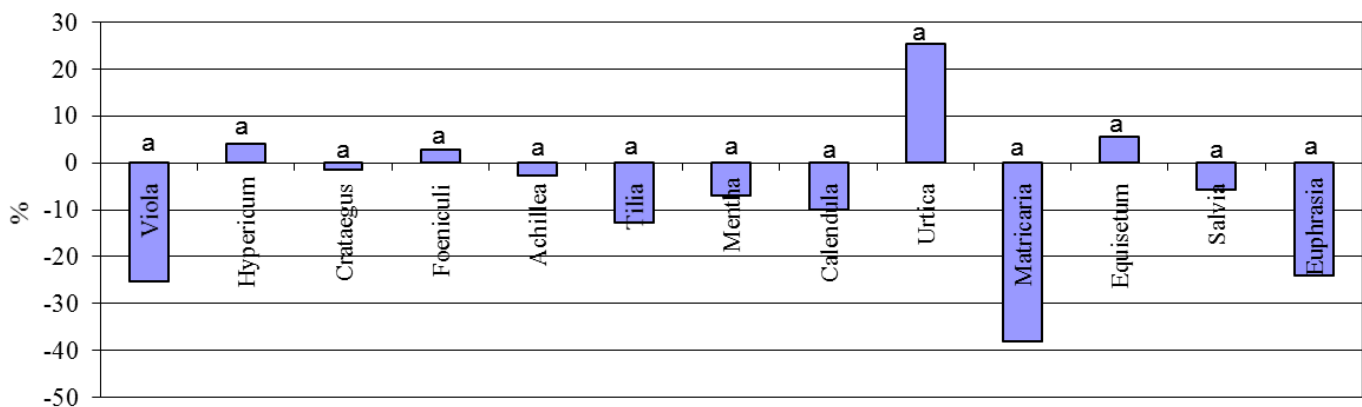
## RESULTS

Assessment carried out after two weeks since the beginning of the experiment demonstrated differences in individual objects concerning seedling emergence. Mean number of buckwheat plants depending on the used substrate ranged from 11 to 22 plants. Most buckwheat plants germinated in the object where *Urtica* sp. leaves were used, while least plants were observed where *Matricaria* sp. inflorescence was applied (Figure 1). In most objects, seedling emergence of common buckwheat did not exceed 50%, apart from those where *Euphrasia* sp. (45%), *Viola* sp. (44%) and *Matricaria* sp. inflorescences (37%) were used as a substrate. In control, seedling emergence of common buckwheat amounting to 60% was noted. A reduction in seedling emergence in comparison with control for the combinations with the lowest noted percentage of seedling emergence was as follows: 38% (*Matricaria* sp.), 25% (*Viola* sp.) and 24% (*Euphrasia* sp.). However, the highest percentage increase when compared to control was noted as a result of using *Urtica* sp. leaves (25%). Nazir et al. (2007) in their study demonstrated an adverse effect of used medicinal plants, that is, *Rheum emondi*, *Saussurea lappa* and *Potentilla fulgens*, on germination of common buckwheat nutlets.

The greatest fresh matter of common buckwheat was obtained from the objects where *Urtica* sp. leaves served as a planting substrate for nutlets (Figure 2). Additionally, equally high values of fresh matter of common buckwheat were obtained after using *Equisetum* sp., *Calendula* sp.,

**Table 1.** Characterization of the tested medicinal plants.

Name		Botanical family/	Used part of plant
English	Latin	Latin name	
Pansy	<i>Viola sp.</i>	Violaceae	Herb
St John'swort	<i>Hypericum sp.</i>	Hypericaceae	Herb
Hawthorn	<i>Crataegus sp.</i>	Rosaceae	Inflorescence
Fennel	<i>Foeniculi sp.</i>	Umbelliferae	Fruit
Yarrow	<i>Achillea sp.</i>	Compositae	Herb
Linden	<i>Tilia sp.</i>	Tiliaceae	Inflorescence
Mint	<i>Mentha sp.</i>	Labiatae	Leaves
Marigold	<i>Calendula sp.</i>	Compositae	Inflorescence
Nettle	<i>Urtica sp.</i>	Urticaceae	Leaves
Chamomile	<i>Matricaria sp.</i>	Compositae	Inflorescence
Horsetail	<i>Equisetum sp.</i>	Equisetaceae	Herb
Sage	<i>Salvia sp.</i>	Labiatae	Leaves
Euphrasia	<i>Euphrasia sp.</i>	Scrophulariaceae	Herb

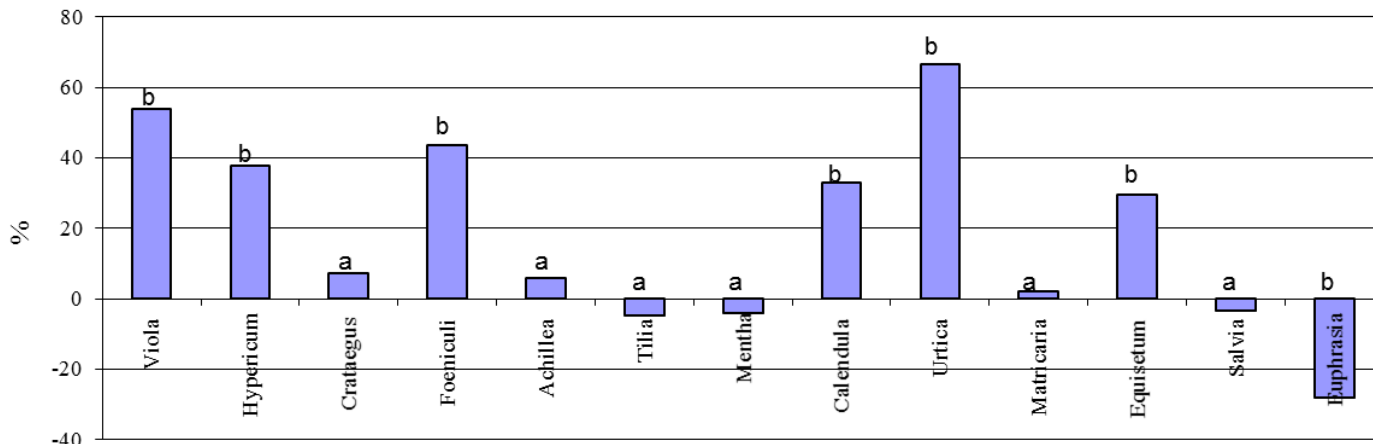


**Figure 1.** Effect of the medicinal plants on the buckwheat germination (increase/decrease comparing to control). a – insignificant change, b – significant change.

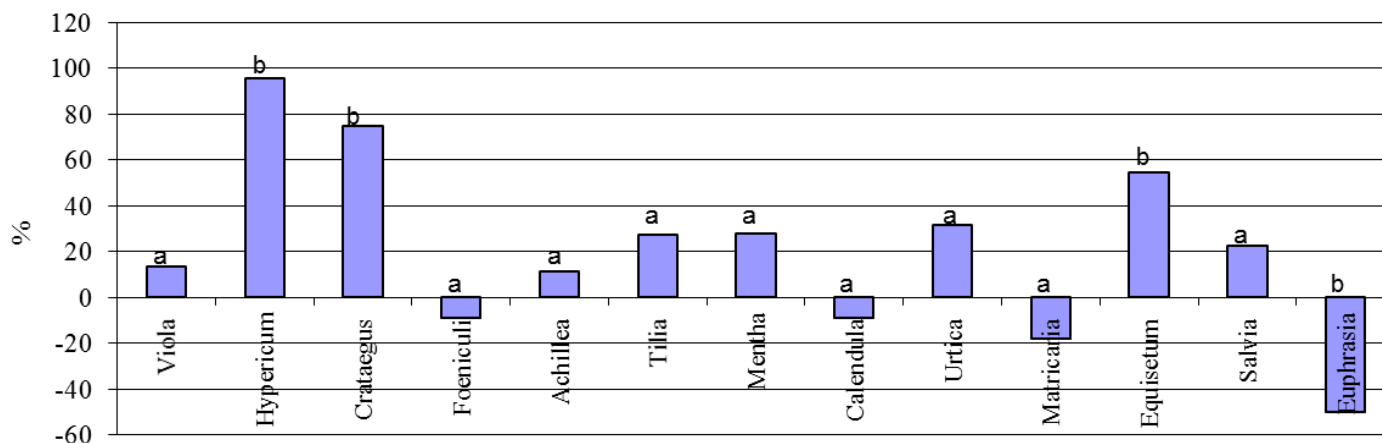
*Hypericum sp.*, *Foeniculi sp.* and *Viola sp.* An increase in fresh matter expressed as a percentage in comparison with matter of buckwheat plants obtained from control amounted to: 30% (for *Equisetum sp.*), 33% (*Calendula sp.*), 38% (*Hypericum sp.*), 44% (*Foeniculi sp.*), 54% (*Viola sp.*) and 66% (*Urtica sp.*). The highest reduction in growth on the basis of plant matter was observed in the object where *Euphrasia sp.* herb was used (by 28% when compared to control). Buckwheat fresh matter from that combination was significantly lower than plant matter from the objects where *Viola sp.*, *Hypericum sp.*, *Crataegus sp.*, *Foeniculi sp.*, *Calendula sp.*, *Urtica sp.* and *Equisetum sp.* were used. Different influence of the tested medicinal plants was observed for opium poppy (Figure 3). Seedling emergence of this crop was strongly reduced by the use of *Euphrasia sp.*, and the percentage

of germinated seeds amounted to only 18%. *Hypericum sp.* had the most favorable effect on poppy plants as it caused 72% of seedling emergence. Apart from the mentioned combination, also, the following plants had a positive influence on poppy germination: *Crataegus sp.* (64% of seedling emergence) and *Equisetum sp.* (57% of seedling emergence). These were the only objects where the percentage of germinated seeds exceeded 50%. Relatively, the lowest percentage of seedling emergence from 18 to 33% was observed after using *Euphrasia sp.*, *Matricaria sp.* and *Foeniculi sp.* The number of germinated seeds in control in the experiment was 11, that is, 37%. The use of *Euphrasia sp.* resulted in seedling emergence of poppy plants reduced by 50% in comparison with control.

The highest fresh matter of opium poppy was obtained



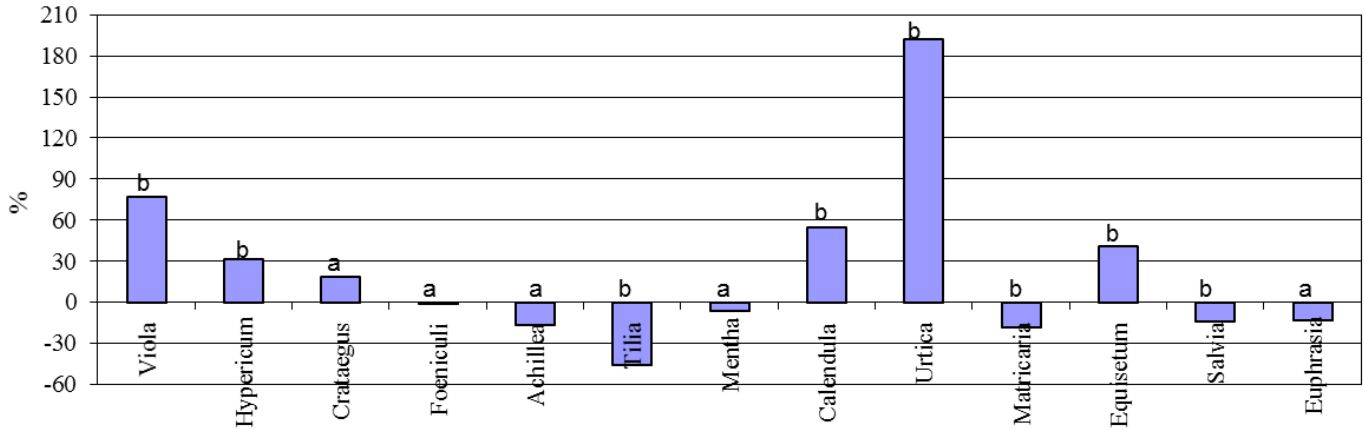
**Figure 2.** Effect of the medicinal plants on the fresh mass of buckwheat (increase/decrease comparing to control). a – insignificant change, b – significant change.



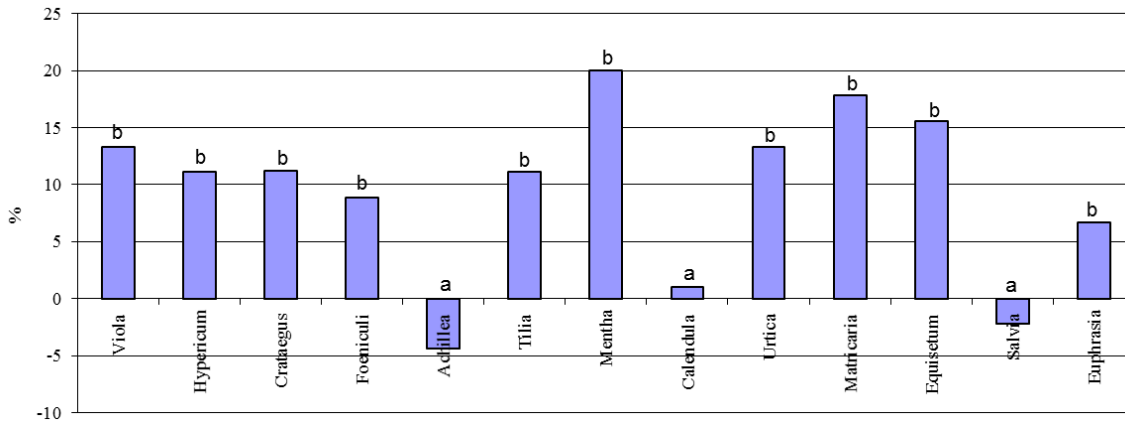
**Figure 3.** Effect of the medicinal plants on the opium poppy germination (increase/decrease comparing to control). a – insignificant change, b – significant change.

in the combination where *Urtica sp.* were used (on average 7.85g), while the lowest was observed where *Tilia sp.* was applied (on average 1.46 g) (Figure 4). An increase in poppy fresh matter in comparison with control after use of *Urtica sp.* amounted to as much as 192%, and a reduction in matter resulting from the use of *Tilia sp.* was 46%. Significantly, higher values of poppy fresh matter when compared to control were noted for *Urtica sp.*, *Calendula sp.* and *Viola sp.*, respectively, by 191, 54.7 and 77%. Plants characterized by the lowest matter (after using *Tilia sp.* inflorescence) were significantly different from the objects where planting substrate included *Viola sp.*, *Hypericum sp.*, *Crataegus sp.*, *Foeniculi sp.*, *Calendula sp.* and *Equisetum sp.* from 82 to 225%. Oilseed rape cultivar Californium and semi-dwarf hybrid Maximus exhibited different response to the

examined medicinal plants. Comparison of germination of both oilseed rape cultivars showed significant differences in seed susceptibility to allelopathins contained in medicinal plants. Most of the examined species had a positive influence on germination of oilseed rape cv. Californium (Figure 5). The greatest effect of germination stimulation for this cultivar in comparison with control was obtained in the object where *Mentha sp.* was used (20%), then *Matricaria sp.* (18%), *Equisetum sp.* (16%), *Viola sp.* and *Urtica sp.* (13%), *Hypericum sp.*, *Crataegus sp.* and *Tilia sp.* (11%). More germinations of 7 and 9%, respectively were obtained for *Euphrasia sp.* and *Foeniculi sp.* in comparison with control. No influence of *Calendula sp.* on germination of this cultivar was noted, while *Achillea sp.* and *Salvia sp.* had an inhibitory influence on seedling emergence of the cultivar. The highest increase in fresh



**Figure 4.** Effect of the medicinal plants on the fresh mass of opium poppy (increase/decrease comparing to control). a – insignificant change, b – significant change.

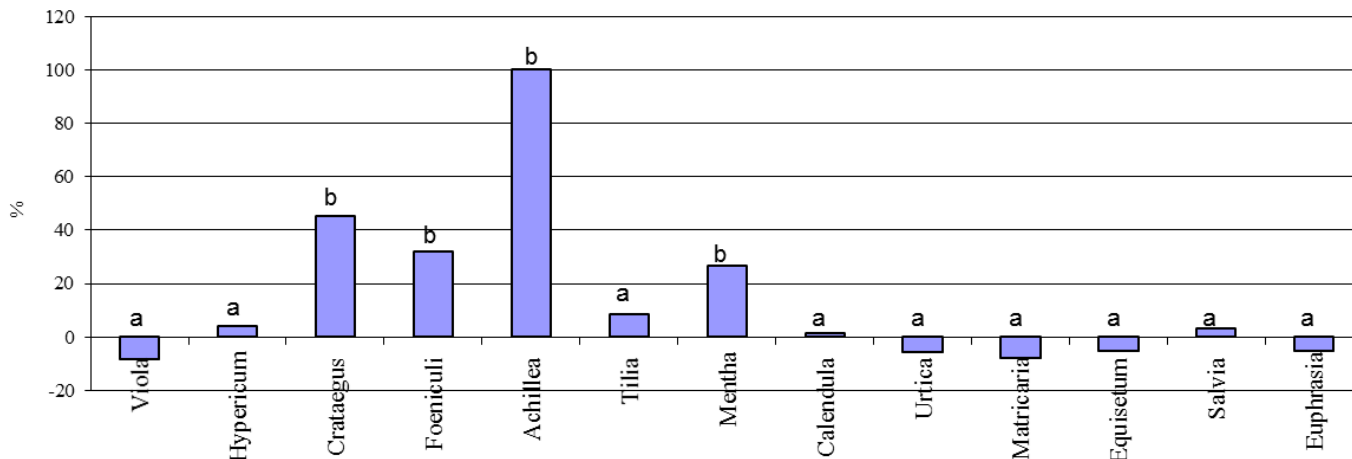


**Figure 5.** Effect of the medicinal plants on the traditional oilseed rape cv. Californium germination (increase/decrease comparing to control). a – insignificant change, b – significant change.

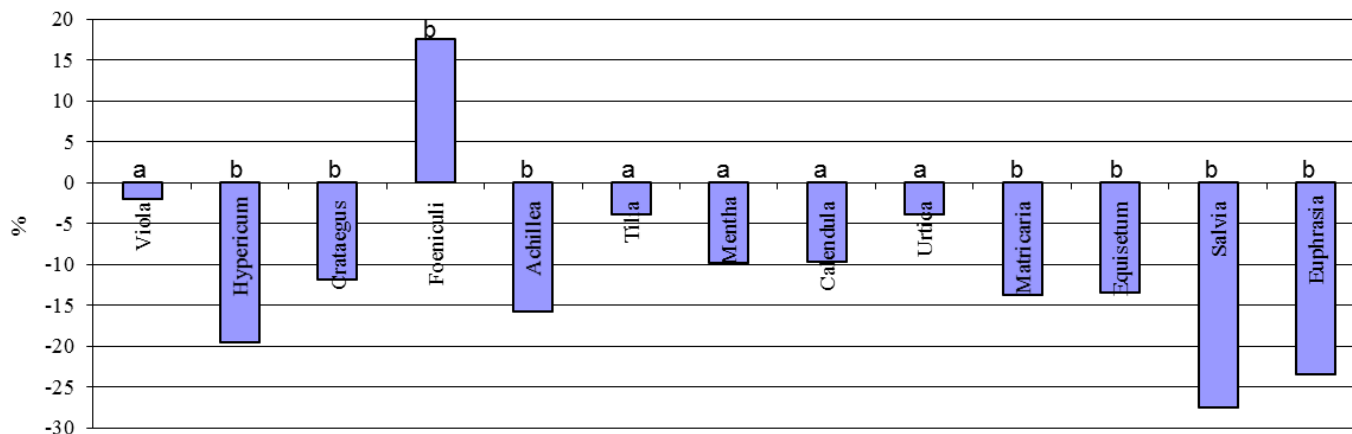
fresh matter was observed after the use of *Achillea sp.*, over 100%, when compared to control (the species had a poor inhibitory effect on germination) (Figure 6). A significant increase in fresh matter of the plants was noted also in the objects where *Crataegus sp.* (45%), *Foeniculi sp.* (32%) and *Mentha sp.* (27%) were used. *Calendula sp.*, *Hypericum sp.* and *Tilia sp.* (2 to 9%) had a lesser influence on an increase in plant matter. The other medicinal plants adversely affected plant matter. The greatest reduction in fresh matter was observed after using *Viola sp.* and *Matricaria sp.* (8%).

The experiments carried out on hybrid semi-dwarf cultivar of oilseed rape (Maximus) indicated an ambiguous effect of the examined medicinal plants on oilseed rape plants because most of them had a different influence on the examined traits. All the medicinal plants inhibited germination of semi-dwarf cultivar of oilseed

rape (Figure 7). The germination was the most reduced by *Salvia sp.* and *Euphrasia sp.* The experimental objects were characterized by a reduction in the number of germinated seeds, respectively by 28 and 24% in comparison with control. In these experimental objects also, the greatest reduction in plant fresh matter was noted (28 to 30%). *Hypericum sp.* inhibited oilseed rape germination by 20% and at the same time increased the level of plant fresh matter by 23%. Fruit of *Foeniculi sp.* and *Achillea sp.* inhibited germination by 16 to 18%, increasing plant matter by 35 to 43%. A similar effect was exhibited by species *Crataegus*. Inflorescence of this plant stimulated oilseed rape germination (increase by 12%), and increased plant matter by nearly 32%. The use of *Matricaria sp.* and *Equisetum sp.* resulted in 14% less germinated seeds when compared to control. Also *Matricaria sp.* had an inhibitory effect on oilseed rape



**Figure 6.** Effect of the medicinal plants on the fresh mass of traditional oilseed rape cv. Californium (increase/decrease comparing to control) a – insignificant change, b – significant change.



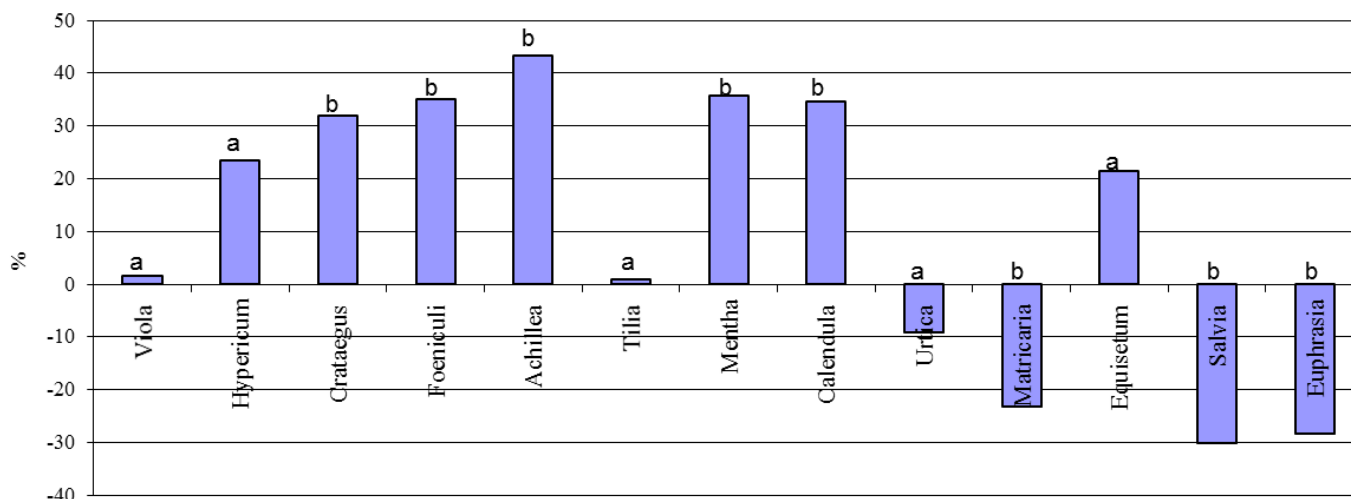
**Figure 7.** Effect of the medicinal plants on the semi-dwarf oilseed rape cv. Maximus germination (increase/decrease comparing to control) a – insignificant change, b – significant change.

growth (reduction in fresh matter by 23%), *Equisetum sp.* stimulated growth of the cultivated plants (increase in matter by 21%) (Figure 8). *Urtica sp.* and *Tilia sp.* (germination reduction by 4%) and *Viola sp.* herb (germination reduction by 2%) reduced germination of oilseed rape cultivar Maximus to a lesser degree. These species did not have a statistically significant influence on an increase or a reduction in fresh matter.

## DISCUSSION

Allelopathic compounds have diverse effects but the most common one described in literature is the influence on seed germination as well as on growth and development of seedlings (Gniazdowska et al., 2004). Experiments assessing influence of sage extract (*Salvia officinalis*) on

germination of two weeds (*Amaranthus retroflexus* and *Portulaca oleraceae*) were conducted by Aziz and Fuji (2006). The authors showed an inhibitory effect of the extract on germination of only one of the mentioned species, *Amaranthus retroflexus*, while Arminante et al. (2006) in their research noted an adverse influence of *S. officinalis* on germination and growth of three plants: *Raphanus sativus*, *Lactuca sativa* and *Lepidium sativum*. Our experiments showed varied response of common buckwheat opium poppy to the used medicinal plants. The greatest stimulatory effect on seedling emergence of common buckwheat exhibited *Urtica* leaves, while seedling emergence of opium poppy was most affected by *Hypericum*. Seedling emergence of common buckwheat was reduced by the use of *Matricaria* inflorescence, while *Euphrasia* herb reduced emergence of field poppy. *Urtica*



**Figure 8.** Effect of the medicinal plants on the fresh mass of semi-dwarf oilseed rape cv. Maximus (increase/decrease comparing to control) a – insignificant change, b – significant change.

leaves caused a significant increase in fresh matter of the common buckwheat and opium poppy. The greatest reduction in fresh matter of buckwheat was noted as a result of using *Euphrasia* herb, while in the case of field poppy, fresh matter was most reduced by using *Tilia* inflorescence. *Mentha sp.* exhibited a strong stimulatory effect on seedling emergence of oilseed rape cultivar Californium, while *Achillea sp.* had an inhibitory influence. All the examined medicinal plants inhibited seedling emergence of hybrid semi-dwarf oilseed rape cv. Maximus.

The study conducted by Habibi Lahigi et al. (2012) confirmed the presence of twenty compounds in *Urtica dioica*, most of them in leaf, in comparison with other plant parts of which neophytadiene, phtaleic acid, dibutylphtaleate, bis (2-ethyl hexyl) maleate and 1,2-benzenocli carboxylic acid were the dominating ones. Fritz et al. (2007) observed germination reduction of *Lactuca sativa* during germination and growth inhibitory effect of *Hypericum myrianthum* and *H. polyanthemum* ethanolic extracts investigation. These researches indicated phenolic compounds as the main allelopathic components while Celen et al. (2008) also revealed presence of tannins as well. Sharopov et al. (2010) identified sixty-six essential oil compounds of *Hypericum perforatum*, with germacrene D,  $\alpha$ -pinene, (E)-caryophyllene, n-dodecanol, caryophyllene oxide, bicyclogermacrene, and spathulenol as the main constituents. Composition of *Matricaria recuita* L. flowers essential oil was assessed by Ghasemi et al. (2013). Essential oil analysis by GC-MS and TLC methods revealed as a main compounds, bisabolol oxide A, bisabolone oxide, bisabolol oxide B, chamazulene, spathulenol and farnesene. Murti et al. (2012), besides

the mentioned substances, also presented sesquiterpens, apigenin, luteolin, quercetin, umbelliferone and en-yndicycloether as chemical components of chamomile. Also, *Euphrasia* contains a lot of chemical compounds represented mainly by phenolic compounds (for exexample, chlorogenic, caffeic acids, quercetin-3-O-rutinoside (9) and apigenin), organic acids (for example, citric acid, quinic acid, acetic acid), sterols (cholesterol,  $\beta$ - Sitosterol) and fatty acids (for example, myrystic, palmitic, docosahexaenoic) determined by different chromatographic methods (Teixeira and Silva, 2013). Spectroscopic techniques performed by Toker et al. (2004) led to isolation of kaempferol 3,7-O- $\alpha$ -L-dirhamnoside (I) and quercetin 3,7-O- $\alpha$ -L-dirhamnoside (II) from the leaves of *Tilia argentea* (Tiliaceae) while two coumarins, one flavan-3-ol, one fatty acid, and two lignan glycosides were isolated by Choi et al. (2008).

Islam and Kato-Noguchi (2013) confirmed allelopathic property of *Mentha* (*Mentha sylvestris*). Authors suggest considering *Mentha* as a potential candidate for isolation and identification of allelochemicals, which were used as a natural herbicides. Sardashti and Adhami (2013) analyzed oils of *Mentha pulegium* L. using gas chromatography/mass spectrometry technique. They recognized 35 constituents of total essential oil, where the pulegone, cineole, isopulegone and beta-pinene were the major ones representing 99.52% of the total essential oil mass from which 29 compounds were elucidated. In other studies (Boukhebt et al. 2011), the major components for *Mentha pulegium* were: pulegone, menthone, pipériténone, pipéritone and isomenthone, limonene and octaan-3-ol. Dias et al. (2013) compared chemical composition of wild and commercial *Achillea millefolium* and they found their profiles similar, but

commercial yarrow have higher content of fat and saturated fatty acids, proteins, ash, energy value, sugars and flavonoids. Bimbiraite et al. (2008) observed the highest content of flavonoids in deep pink morphotype while the highest content of essential oil was found in white morphotype. Literature regarding allelopathy provides examples of plant species characterized by great allelopathic potential towards cultivated species. Some authors emphasize the fact of a change in pH of soil with allelopathic compounds (Khalid et al., 2002). Bhatia et al. (1982) named *Chenopodium album* as a plant specially involved in stimulating wheat germination, and *Chenopodium murale* as a plant stimulating mustard growth. On the other hand, Kossanel et al. (1977) considered *Chenopodium album* as a plant inhabiting germination and growth of corn. Bhowmic and Doll (1984) in their study obtained a positive effect of extracts of *Chenopodium album*, *Amaranthus retroflexus*, *Artemisia artemisifolia*, *Abutilon theophrasti* and *Setaria glauca* on soy and corn. Elmore et al. (1985) stated that *Cyperus rotundus* caused a decrease in yield of cotton, corn, sorghum and tobacco. Plants with a strong inhibitory effect on germination, seedling growth and on an increase in fresh matter of many cultivated plants include also: *Lolium multiflorum*, *Diachanthium annulatum*, *Euphorbia granulata* (Hussain, 1980), *Datura innoxia*, *Citrullis colocynthis*, *Stachys parviflora* (Hussain et al., 1986). Among medicinal plants, strong allelopathic properties can be observed for example, in *Rheum emodi*, *Saussurea lappa* and *Potentilla fulgens*. Nazir et al. (2007) indicated that the plants reduced germination of such cultivated plants as: love-lies-bleeding (*Amaranthus caudatus*), mung bean (*Phaseolus mungo*), common bean (*Phaseolus vulgaris*), finger millet (*Elusine coracana*), common wheat (*Triticum aestivum*) and common buckwheat (*Fagopyrum esculentum*).

## CONCLUSION

This study gives a general outlook of the allelopathic effects of popular medicinal plants on some crops. It has been observed that increase of common buckwheat seeds germination is strongly enhanced by *Urtica sp.* whereas *Matricaria sp.*, *Euphrasia sp.*, and *Viola sp.* have inhibitory effects on that process. The studies revealed that later growth of common buckwheat was positively stimulated by such herbs as *Urtica sp.*, *Viola sp.*, *Foeniculi sp.*, *Colendula sp.* and *Equisetum sp.* Significant increase of opium poppy germination capacity was recorded in the presence of *Hypericum sp.*, even though, *Crataegus sp.* and *Equisetum sp.* have also given positive results. However seed germination of opium poppy was suppressed by *Euphrasia sp.* The maximum stimulatory effect on the traditional cultivar of oilseed rape germination was caused by *Mentha sp.* but

the most tested medicinal plants rape enhanced that process whereas germination capacity of semi-dwarf oilseed rape cultivar increased as a result of *Foeniculi sp.* presence. Further growth and development of traditional oilseed rape was significantly and positively affected by *Mentha sp.*, *Crataegus sp.* and *Foeniculi sp.* but extremely positive results were obtained in the presence of *Achillea sp.* In case of semi-dwarf oilseed rape cultivar, later growth was stimulated by *Achillea sp.*, *Crataegus sp.*, *Foeniculi sp.*, *Mentha sp.* and *Calendula sp.* This paper confirms different response of popular crops on the presence of some medicinal plants. These experiments can be valuable in agronomical practice because some of tested herbs are frequently present on fields and they can significantly reduce yield, and on the other hand some of them can be used as alleloherbicides or natural bio-stimulants in crops.

## Conflict of Interest

We declare that we have no conflict of competing interest.

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Full Length Research Paper

# Nobiletin, a polymethoxylated flavone from citrus peels, induces differentiation of normal human epidermal keratinocytes

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**Nobiletin, a polymethoxylated flavone from citrus peels, is known to have a wide range of pharmacological activities. In this study, we examined the effects of nobiletin on differentiation of normal human epidermal keratinocytes (NHEKs). Treatment of NHEKs with nobiletin was found to cause marked increases in the expression level of keratin 10 (K10) and involucrin, differentiation makers of keratinocytes.**

**Key words:** Nobiletin, polymethoxylated flavone, *citrus*, keratinocyte, keratin.

## INTRODUCTION

The epidermis consists of several cell layers, each of which contains keratinocytes at distinct stages of differentiation. The deepest or basal layer located at the dermal-epidermal junction is composed of undifferentiated keratinocytes that continuously proliferate (Regnier et al., 1986). While migrating upward through the epidermis, keratinocytes undergo extensive differentiation that is essential for the skin to function as a protective barrier (Proksch et al., 1993). Keratinocyte differentiation initiates in the spinous layer (Roop et al., 1983), which is characterized by growth arrest and expression of the keratins 1 (K1) and 10 (K10) proteins. This early differentiation in the spinous layer is followed by late differentiation in the granular layer, which is accompanied by expression of

proteins such as involucrin (Eckert et al., 1993). After terminal differentiation, keratinocytes undergo an epidermal-specific programmed cell death to form the cornified envelope that serves as a barrier to water loss and microbial invasion (Nemes et al., 1999). The envelope contains many proteins, among which involucrin was first discovered and shown to become cross-linked to a cellular transglutaminase (Simon et al., 1985).

However, abnormal differentiation of keratinocytes in epidermis has led to epidermal dysfunction, such as epidermal thinning, barrier dysfunction, and delayed wound healing (Nuccitelli et al., 2011). Therefore, the inducer of keratinocyte differentiation may serve as dermatological agent by normalization of epidermal turnover.

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Nobiletin is one of the most abundant polymethoxylated flavones present in citrus peels. This compound shows various biological and pharmacological activities such as anti-inflammatory (Murakami et al., 2000; Lin et al., 2003), carcinogenic (Morley et al., 2007; Walle et al., 2007; Akao et al., 2008) and allergic effects (Itoh et al., 2008). However, it is not yet known whether nobiletin affects the keratinocyte differentiation. In the present study, we investigated the effects of nobiletin on differentiation of normal human epidermal keratinocytes (NHEKs).

## MATERIALS AND METHODS

### Materials

Nobiletin, sinensetin, 5-demethyl sinensetin and tangeretin were purified from *Citrus reticulata* and their purities were greater than 98% (Iinuma et al., 1980). Each compound was dissolved in DMSO and added to the cell culture medium with a final DMSO concentration of 0.1 % v/v.

### Cell culture

NHEKs were purchased from Kurabo (Osaka, Japan). Cells were cultured in a serum-free keratinocyte growth medium, HuMedia-KB2 (Kurabo, Osaka and Japan), supplemented with bovine pituitary extract (0.4% v/v), human recombinant epidermal growth factor (0.1 ng/ml), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), gentamicin (50 µg/ml) and amphotericin-B (50 ng/ml), at 37°C in a humidified, CO<sub>2</sub>-controlled (5%) incubator.

### Western blot analysis

The expression levels of keratinocyte differentiation-specific markers in NHEKs were analyzed by Western blot analysis. NHEKs were lysed by incubating at 4°C for 30 min in lysis buffer (10 mM Tris-HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing the protease inhibitor mixture (Complete™). After centrifugation of the cell lysates, the supernatant was isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred electrophoretically into a Microporous polyvinylidene difluoride (PVDF) membrane. After blocking in 5% skim milk and 0.05% Tween-20, blots were incubated with either anti-K10 (Lab vision) or -involucrin (Lab vision) antibody, and then further incubated with a horseradish peroxidase-conjugated secondary antibody (GE healthcare). Proteins were visualized using the Enhanced chemiluminescence (ECL) Western blotting detection system and gel images were obtained with the LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

## RESULTS AND DISCUSSION

To examine the effects of nobiletin on epidermal keratinocyte differentiation, we analyzed by Western blot analysis changes in protein expression of K10, an early-stage differentiation marker, as well as involucrin, a late-stage differentiation marker. Calcium is known to be a major factor for triggering the differentiation of cultured keratinocytes (Yuspa et al., 1989). calcium chloride was used as a positive control to induce differentiation. As

shown in Figure 1A, nobiletin treatment at 10 µM for 72 h markedly enhanced the expression of K10 protein (27.4-fold), while K10 induction by the presence of high extracellular calcium (1.2 mM) was only 5.3-fold. Nobiletin also induced involucrin protein expression. However, the involucrin induction by nobiletin (2.8-fold) was lower than that by calcium treatment (8.7-fold). The cornified envelope precursor proteins such as involucrin are expressed later in the keratinocyte differentiation in granular layers of the epidermis. It has been reported that high calcium may propel cultured keratinocytes past early differentiation steps to a later differentiation stage, resulting in a slight reduction in K10 promoter activity (Yuspa et al., 1989). This and our present findings suggest that nobiletin induces keratinocyte differentiation especially early phase differentiation. Nobiletin increased the expression level of K10 in a concentration-dependent manner with a maximum induction at 10 µM (Figure 1B). In addition, the levels of K10 protein increased with increasing incubation time, and maximum induction was seen at 72 h after nobiletin treatment (Figure 1C).

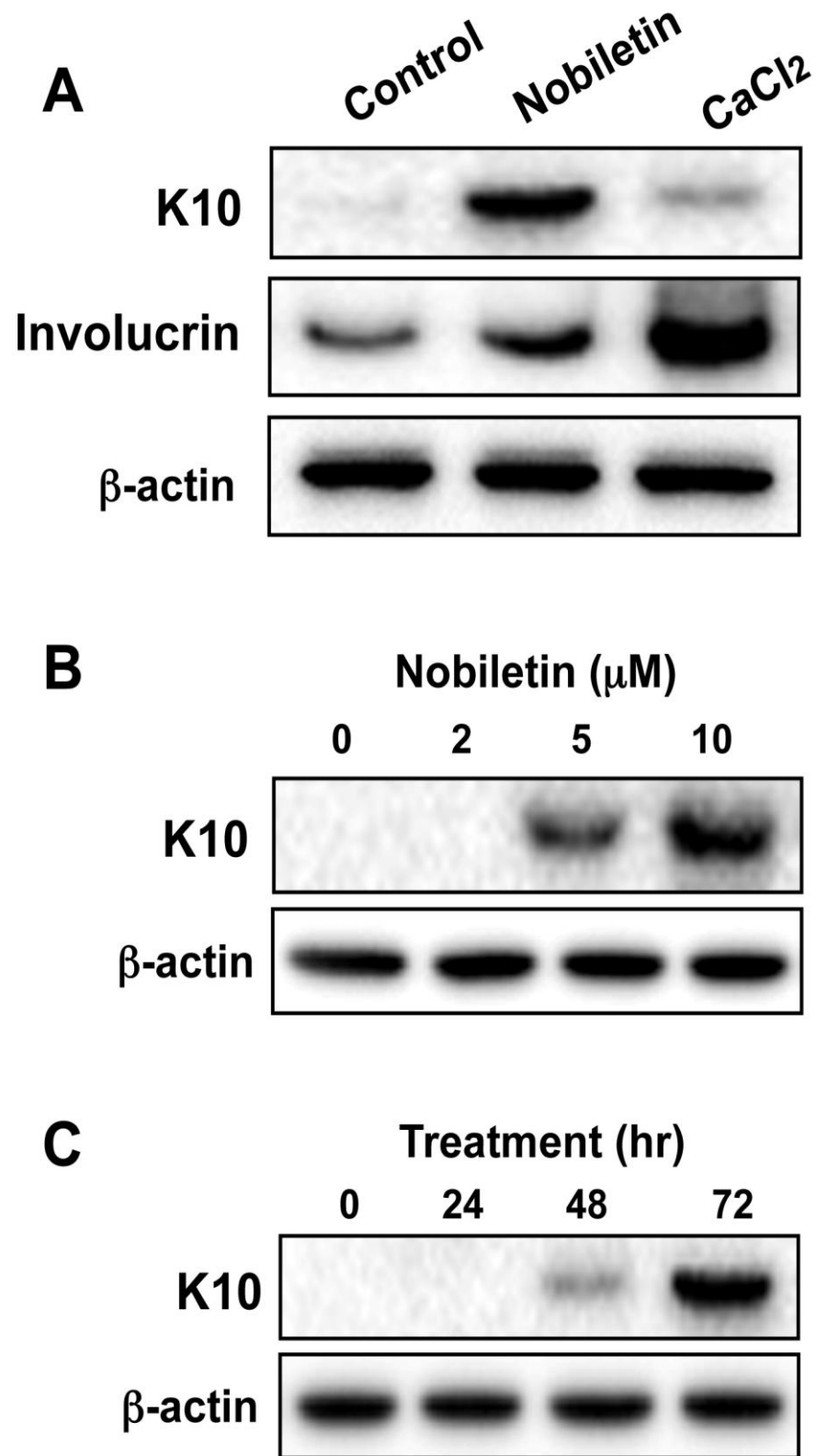
Finally, we examined the effects of other three polymethoxylated flavones of *Citrus* on expression of K10 protein, including sinensetin, 5-demethyl sinensetin and tangeretin. Sinensetin and 5-demethyl sinensetin significantly increased expression levels of K10, but the effects were less than that seen for nobiletin (Figure 2). Intriguingly, tangeretin that differs from nobiletin only by the absence of a methoxyl group on the B-ring exhibited much less effect on induction of K10 protein. The presence of two methoxyl groups on the B ring appears to be critical for the differentiation-inducing effect. Several intracellular signaling pathways have been identified as regulators of keratinocyte differentiation. Phosphatidylinositol 3-kinase (PI3K), nuclear factor kappa B (NF-κB), and extracellular signal-regulated kinase (ERK) are implicated in the early phase of differentiation (Sayama et al., 2002; Liu et al., 2009; Schmidt et al., 2000). It is also known that transcription of K10 gene is regulated by the transcription factor CCAAT/enhancer binding protein β (C/EBPβ (Zhu et al., 1999). It is thus likely that nobiletin induces differentiation by affecting these signaling pathways. Further studies are required to elucidate the exact mechanisms underlying the effects of nobiletin on keratinocyte differentiation, which are in progress in our laboratory.

## Conclusion

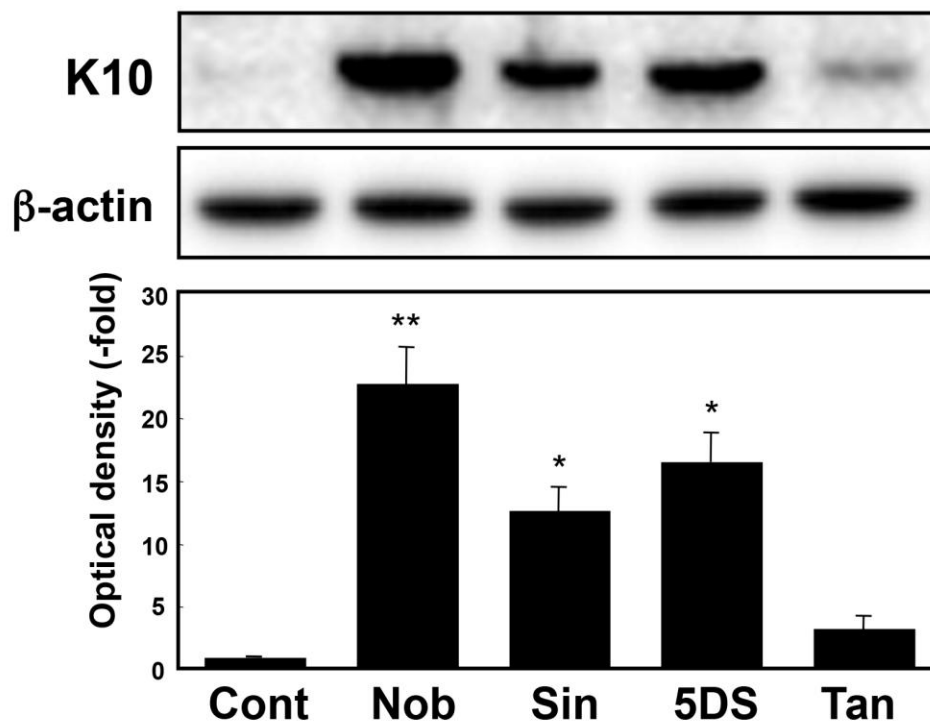
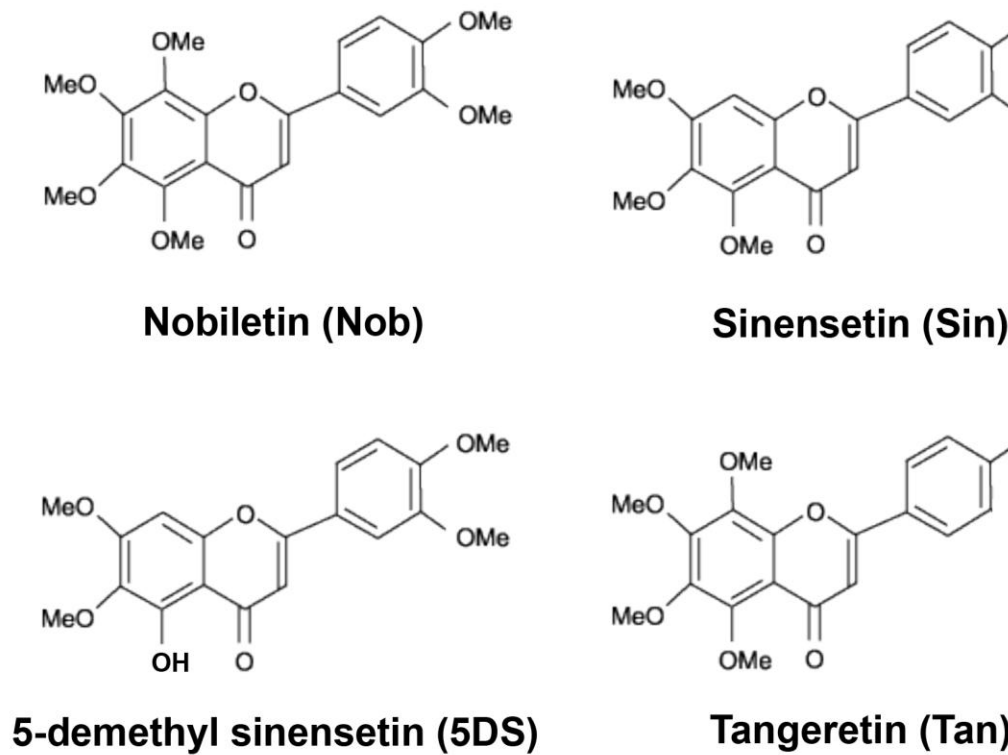
In summary, the present study demonstrated that the ability of nobiletin to induce differentiation of NHEKs suggests a dermatological and cosmetic agent for normalizing epidermal turnover.

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**Figure 1.** Induction of differentiation markers by nobiletin in human epidermal keratinocytes. The levels of K10, involucrin and  $\beta$ -actin as an internal loading control in total cell lysates were analyzed by Western blot analysis. A representative blot of three independent experiments is shown.



**Figure 2.** Effects of various polymethoxylated flavones on expression of K10 in human epidermal keratinocytes. The levels of K10 and  $\beta$ -actin as an internal loading control in total cell lysates were analyzed by Western blot analysis. A representative blot of three independent experiments is shown. Data represent the mean  $\pm$  S.D. of three independent experiments. Asterisks indicate statistical significance as determined by Student's *t* test (\*  $p < 0.05$ , \*\*  $p < 0.01$  vs. Cont).

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### Conflicts of Interest

All authors report no conflict of interest.

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