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SHORT COMMUNICATION

Phenolics in the *Tussilago farfara* leaves

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Summary

Coltsfoot leaves (*Farfarae folium*) are used in the European medicine in respiratory tract diseases, for cough, bronchitis and asthmatic disorders, while in the traditional Chinese medicine only flower buds (*Farfarae flos*) have been recognized as a medicine. A short literature review shows that most data concern the chemical composition of the coltsfoot flowers. During the carried out studies we have isolated and identified (UV, ^{1}H and ^{13}C NMR, analysis of acid and enzymatic hydrolyze products) six known flavonols from the coltsfoot leaves: kaempferol and its 3-*O*-*β*-glucopyranoside and 3-*O*-*α*-rhamnopyranosyl(1→6)-*β*-glucopyranoside, along with quercetin derivatives: 3-*O*-*β*-arabinopyranoside, 3-*O*-*β*-glucopyranoside and 3-*O*-*α*-rhamnopyranosyl(1→6)-*β*-glucopyranoside. Moreover, we have detected the presence of three phenolic acids.

Key words: *Tussilago farfara, leaves, flavonoids, phenolic acids*

INTRODUCTION

Tussilago farfara L., known as coltsfoot, is a perennial herbaceous plant from *Asteraceae* family, common in Europe and Asia, as well as in the North and South Americas. The yellow flowers appear in early spring, whereas leaves are set after the seeds. In China the coltsfoot flower buds are an important traditional medicine and have been used for centuries and also contemporarily, to relieve respiratory tract aliments, mainly as a cough suppressant, expectorant, as well as a soothing agent for mucosa, skin diseases, wounds and pimples.

The literature data regarding the coltsfoot chemical constituents concern mainly its flower buds. The extensive phytochemical studies have revealed that *Farfarae flos* contains a number of diverse components including flavonoids [1-7], simple phenolic acids [2, 3, 6, 7] along with caffeic acid derivatives [1, 2, 6, 7], sesquiterpenes (mostly tussilagone) [6-12], chromones [13], essential oil [14], phytosterols, fatty acids, amino acids, inorganic acids [6, 7] and pyrrolizidine alkaloids [15]. Some of the pyrrolizidine alkaloids (e.g. senkirkine and senecionine) due to a long-term consumption, can be hepatotoxic [16], causing damage to the liver or even a liver cancer in severe cases. Due to small quantities of pyrrolizidine alkaloids present in the plant material, the risk of toxicity is rather low.

Sesquiterpenoids seem to be one of the major pharmacologically active compounds of *Farfarae flos*. One of them, tussilagone, may be of significances in the treatment of cardiovascular and respiratory systematic diseases (e.g. asthma), acting as a weak platelet activating factor antagonist and a Ca^{2+} channel blocker [17]. The extracts of *Farfarae flos* exhibit various activities, such as antioxidant [18, 19], anti-inflammatory [20] and antimicrobial [21-23]; what is more, they also inhibit some enzymes including α-glucosidase [24], NO synthetase in the LPS-activated macrophages or murine microglial cells [8, 25] and diacylglycerol acyltransferase [26].

In European medicine, the coltsfoot leaves from the plant varieties devoid of the hepatotoxic pyrrolizidine alkaloids are used in the same cases like flower buds in China. In Poland either the coltsfoot leaves or flowers are available as a single herb for preparation of herbal teas.

Even though coltsfoot leaves are used in medicine as an ingredient of different herbal preparations (herbal teas, syrups, tablets) for upper respiratory tract infections with cough and difficulties in expectorations, there is no data concerning its chemical constituents. Thus, the aim of our studies was isolation and identification of the flavonoids present in the coltsfoot leaves.

MATERIAL AND METHODS

Plant material

The leaves of *Tussilago farfara* L. were purchased from the Zakład Zielarski Kawon, Gostyń, Poland (Lot No. 1066.2011).

Extraction and isolation

The dried leaves (150 g) were macerated three times with cold MeOH, next with boiling MeOH and finally with boiling 50% MeOH. Then, the extracts were concentrated under the vaccuo to a syrupy consistency of 17 g, 20 g and 22 g, respectively. Each extract separately was treated with hot H_2O, filtered and chromatographed (PC) in S_1-HOAc-H_2O (15:85). The combined filtrates were extracted successively with

$CHCl_3$ to remove ballast substances, then with Et_2O and EtOAc. The **Et_2O** extract was chromatographed (CC) on a Sephadex LH-20 column with MeOH as an eluent, yielding fraction **I**, containing free phenolic acids, and fraction **II** containing compound **A**.

The **EtOAc** extract was chromatographed (CC) on a cellulose column run with S_7 as an eluent, yielding 90 fractions. After the chromatographic analysis fractions: (5-7), (25-39), and (65-73) were separated by preparative paper chromatography (PC, Whatman No.3, S_1) to yield compound **A** from the fractions (5-7), compounds **B, C, D**, and **E** from the fractions (25-39), and **C, D, E, F** from the fractions (65-73). The individual compounds were finally cleaned-up by CC on Sephadex LH-20 in S_8.

Identification

Chromatography, solvent systems

PC Whatman No.1 or 3: S_1-HOAc-H_2O (15:85); S_2-*n*-BuOH-HOAc-H_2O (4:1:2), S_3*iso*-PrOH-HCO_2H 85%-H_2O (2:5:5), S_4-EtOAc-HCO_2H 85%-H_2O (10:2:3) organic phase,

TLC DC-Alufolien, cellulose, Merck: S_5-*n*-PrOH-EtOAc-H_2O (7:2:1)

2D-TLC DC-Alufolien, cellulose: 1st direction S_6-C_6H_5Me-HOAc-H_2O (6:7:3) organic phase; 2nddirection S_1

CC. Cellulose: S_7- EtOAc –MeOH-H_2O (100:6:20)

CC. Sephadex LH-20 (Pharmacia, Uppsala): S_8-MeOH

Visualization reagents

Flavonoids: 1% methanolic $AlCl_3$, 0.1% methanolic Naturstoffreagenz A (NA reagent), UV_{366nm}.

Sugars: aniline phthalate and heating at 105°C, VIS.

Phenolic acids: UV_{366nm}, diazotized sulphanilic acid and 20% Na_2CO_3 (1:1), VIS.

Partial acid hydrolysis: 1 mg of compounds **E** or **F** was heated in 1 ml of 0.5% HCl. After 15 min. the process was stopped. The obtained hydrolyzates were extracted with EtOAc and the organic phases were separated by preparative chromatography (PC, Whatman No.1 S_1). The bands corresponding to the intermediate products were eluted and subjected to UV spectral analysis and complete hydrolysis.

Enzymatic hydrolysis: 2 mg of compounds **C** or **D** were treated with 1 mg of *β*-glucosidase (Koch-Light) in 1 ml of H_2O at a room temperature till the process was completed (the progress of hydrolysis was monitored by PC in S_1). The hydrolyzates were extracted with EtOAc to obtained aglycones, whereas the sugars remained in the water layers.

Total acid hydrolysis: 1 mg of compounds **B**, **C**, **D**, **E** or **F** and the intermediate products obtained in partial acid hydrolyses were heated in 1 ml of 1% HCl for 1 h. Then the hydrolyzates were extracted with EtOAc.

The aglycones present in the organic phases were identified by co-chromatography (PC S2, S3), whereas the water layers were analyzed for sugars (TLC, S4).

Standards

Flavonoid compounds previously isolated from different species of plants in the Pharmacognosy Department, Poznań University of Medical Sciences (Poland), Sugars: Merck.

Spectral analysis: UV spectra were recorded on a Perking Elmer Lambda 35 UV/VIS Spectrometer, in methanol, before and after the addition of the shift reagents according to Mabry (6). The ^{1}H NMR (300 MHz) and ^{13}C NMR spectra were taken on a Varian Unity 300 MHz spectrometer in DMSO-d_6 solution, with tetramethylsilane (TMS) as internal standard.

RESULTS AND DISCUSSION

The Et_2O and EtOAc fractions were obtained from the crude *Tussilago farfara* L. leaves extract preliminary purified with chloroform. Separation of the Et_2O extract on a Sephadex LH-20 column eluted with methanol yielded subfractions **I** and **II**. Comparison of the studied subfraction with the standards (i.e. the R_f values, fluorescence under UV_{366nm} and spot colours before and after visualization with a mixture of diazotized sulphanilic acid and Na_2CO_3) showed that the subfraction **I** contained *p*-hydroxybenzoic, *cis-, trans- p*-coumaric, and *cis-, trans*- chlorogenic acids, whereas from the subfraction II, compound **A** was isolated and identified as kaempferol. Moreover, five known flavonoids (**B**, **C**, **D**, **E** and **F)** were isolated from the EtOAc fraction. Identification of all compounds was carried out by chromatographic analysis as well as of their hydrolysis products, co-chromatography with the standards, followed by the UV, ^{1}H and ^{13}C NMR spectroscopies.

Table 1.

A comparison of flavonoids from leaves of *Tussilago farfara* according to literature data*

Flavonoids	*Farfarae folium*	*Farfarae flos**	References
Aglycones			
Kaempferol	+	+	1, 2, 4
Quercetin	–	+	1, 2, 3, 4
Kaempferol glycosides			
3-*O*-*β*-D-Glucopyranoside (astragalin)	+	+	1, 3, 4
3-*O*-*β*-L-Arabinopyranoside	–	+	4
3-*O*-*a*-L-Rhamnopyranosyl(1→6)-*β*-D-Glucopyranoside (nicotiflorin)	+	+	2
Quercetin glycosides			
4'-*O*- *β*-D-Glucopyranoside	–	+	2, 4

Flavonoids	*Farfarae folium*	*Farfarae flos*°	References
3-*O*-*β*-L-Arabinopyranoside (guajaverin)	+	+	4
3-*O*-*β*-D-Glucopyranoside (isoquercitrin)	+	+	2
3-*O*-*β*-D-Galactopyranoside (hyperoside)	–	+	1, 2, 3, 5
3-*O*-*α*-L-Rhamnopyranosyl(1→6)-*β*-D-glucopyranoside (rutin)	+	+	1, 2, 3, 5, 6, 7

Kaempferol [A]: PC R_f S_1=0.05, yellow needles; UV λ_{max} (nm): MeOH 266, 298sh, 367; +NaOAc 275, 310sh, 387; +NaOAc/H_3BO_3 267, 372; +NaOMe 279, 323sh, 416; +$AlCl_3$ 268, 324, 424; +$AlCl_3$/HCl 269, 324, 424; UV λ_{max} (nm): ^{1}H-NMR (DMSO-d_6+D_2O) δ (ppm): 6.24 (d, *J*=2.0, H-6), 6.35 (d, *J*=2.0, H-8), 7.94 (d, *J*=8.6, H-2', 6'), 6.88 (d, *J*=8.6, H-3', 5').

Quercetin 3-*O*-*β*-arabinopyranoside [B]: PC R_f S_1=0.30, yellow needles; total acid hydrolysis: quercetin and arabinose; UV λ_{max} (nm): MeOH 258, 298sh, 357; +NaOAc 272, 319, 383; +NaOAc/H_3BO_3 262, 377 ; +NaOMe 274, 320sh, 403; +$AlCl_3$ 276, 302sh, 430; +$AlCl_3$/HCl 271, 301sh, 360, 401.

Quercetin 3-*O*-*β*-glucopyranoside [C]: PC R_f S_1=0.38, yellow needles, total acid and enzymatic hydrolysis: quercetin and glucose, UV λ_{max} (nm): MeOH 257, 298sh, 359; +NaOAc 270, 322sh, 384; +NaOAc/H_3BO_3 262, 377; +NaOMe 273, 328sh, 411; +$AlCl_3$ 274, 333sh, 434; +$AlCl_3$/HCl 268, 361, 399; ^{1}H-NMR (DMSO-d_6+D_2O) δ (ppm): 6.20 (d, *J*=1,9 Hz, H-6), 6.40 (d, *J*=2.0 Hz, H-8), 7.59 (d, *J*=2.2 Hz, H-2'), 6.85 (d, *J*=8.5 Hz, H-5'), 7.57 (dd, *J*=2.5/8.5 Hz, H-6'), 5.46 (d, *J*=7.5 Hz, H-1" glucose); ^{13}C NMR (DMSO-d_6) δ (ppm):156.1 (C-2), 133.3 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.3 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.2 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 116.2 (C-5'), 121.6 (C-6'), 100.8 (C-1"), 74.1 (C-2"), 76.5(C-3"), 69.9 (C-4"), 77.5 (C-5"), 60.9 (C-6").

Kaempferol 3-*O*-*β*-glucopyranoside [D]
PC R_f S_1=0.45, yellow needles; total acid and enzymatic hydrolysis: kaempferol and glucose; UV λ_{max} (nm): MeOH 266, 299sh, 349; +NaOAc 274, 304, 376; +NaOAc/H_3BO_3 267, 303sh, 353; +NaOMe 257, 325, 401; +$AlCl_3$ 274, 304, 350, 397; +$AlCl_3$ /HCl 275, 302, 345, 396; ^{1}H-NMR (DMSO-d_6+D_2O) δ (ppm): 6.20 (d, *J*=2.0 Hz, H-6), 6.43 (d, *J*=2.0 Hz, H-8), 8.04 (d, *J*=8.8 Hz, H-2', 6'), 6.88 (d, *J*=8.8 Hz, H-3', 5'), 5,44 (d, *J*=7,5 Hz, H-1" glucose); ^{13}C NMR (DMSO-d_6) δ (ppm):156.3 (C-2), 133.0 (C-3), 177.8 (C-4), 160.9 (C-5), 98.8 (C-6), 163.5 (C-7), 94.6 (C-8), 156.3 (C-9), 104.4 (C-10), 121.9 (C-1'), 130.7 (C-2'), 115,0 (C-3'), 159.0 (C-4'), 115.0 (C-5'), 130.7 (C-6'), 101.0 (C-1"), 74.0 (C-2"), 77.5 (C-3"), 70.5 (C-4"), 76.3 (C-5"), 61.0 (C-6").

Quercetin 3-*O*-*a*-rhamnopyranosyl (1→6) - *β*- glucopyranoside [E]
PC R_f S_1=0.49, yellow amorphous powder; total acid hydrolysis: quercetin, glucose, rhamnose, partial acid hydrolysis: quercetin 3-O-glucoside as a secondary heteroside, chromatographically identical with compound C. UV λ_{max} (nm): MeOH 258, 299sh, 359; +NaOAc 270, 324, 396; +NaOAc/H_3BO_3 263, 383; +NaOMe 272, 328, 413; +$AlCl_3$ 274, 302sh, 429; +$AlCl_3$ /HCl 270, 300sh, 364, 401; (DMSO-d_6+D_2O) δ (ppm): 6.19 (d, *J*=2.0 Hz, H-6), 6.40 (d, *J*=2.0 Hz, H-8), 7.51 (d, *J*=2.0 Hz, H-2'), 6.85 (d, *J*=8.3 Hz, H-5'), 7.53 (dd, *J*=2.1/8.3 Hz, H-6'), 5.31 (d, *J*=7.5 Hz, H-1" glucose), 4.37 (d, *J*=1.2 Hz, H-1"' rhamnose), 0.96 (d, *J*=6.2 Hz, H-6"' rhamnose); ^{13}C NMR (DMSO-d_6) δ (ppm):156.4 (C-2), 133.3 (C-3), 177.3 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.6 (C-8), 156.6 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.2 (C-2'), 144.7 (C-3'), 148.4 (C-4'), 116.2 (C-5'), 121.6 (C-6'), 101.2 (C-1"), 74.0 (C-2"), 75.9 (C-3"), 70.0 (C-4"), 76.4 (C-5"), 66.9 (C-6"), 100.7 (C-1"'), 70.3 (C-2"'), 70.5 (C-3"'), 71.8 (C-4"'), 68.23 (C-5"'), 17.7 (C-6"').

Kaempferol 3-*O*-*a*-rhamnopyranosyl (1→6)- *β*- glucopyranoside [F]
PC R_f S_1=0.52, yellow needles; total acid hydrolysis: kaempferol, glucose, rhamnose; partial acid hydrolysis: kaempferol 3-O-glucoside as a secondary heteroside, chromatographically identical with compound D. UV λ_{max} (nm): MeOH 267, 352; +NaOAc 279, 378; +NaOAc/H_3BO_3 270, 350; +NaOMe 270, 400; +$AlCl_3$ 277, 303, 348, 400; +$AlCl_3$ /HCl 277, 300, 345, 400. ^{1}H-NMR (DMSO-d_6+D_2O) δ (ppm): 6.35 (d, *J*=2.0 Hz, H-6), 6.65 (d, *J*=2.0 Hz, H-8), 8.20 (d, *J*=8.6 Hz, H-2', 6'), 7.03 (d, *J*=8.8 Hz, H-3', 5'), 5.20 (d, *J*=8.0 Hz, H-1" glucose), 4.50 (brs, H-1"' rhamnose), 1.00 (d, *J*=5.0 Hz, H-6"' rhamnose); ^{13}C NMR (DMSO-d_6) δ (ppm):153.6 (C-2), 132.9 (C-3), 177.2 (C-4), 161.1 (C-5), 98.1 (C-6), 163.8 (C-7), 93.5 (C-8), 155.9 (C-9), 104.2 (C-10), 120.7 (C-1'), 129.7 (C-2'), 115.0 (C-3'), 159.6 (C-4'), 115.0 (C-5'), 130.2 (C-6'), 101.5 (C-1"), 74.2 (C-2"), 76.0 (C-3"), 70.5 (C-4"), 74.8 (C-5"), 66.7 (C-6"), 100.1 (C-1"'), 71.0 (C-2"'), 70.0 (C-3"'), 71.5 (C-4"'), 68.3 (C-5"'), 17.5 (C-6"').

On the TLC or PC chromatograms, in UV light, all isolated flavonoids except from **A,** appeared as dull brown spots. **D** and **F** changed to yellow with Naturstoffreagenz A which indicated the presence of free 5- and 4´-hydroxyl groups and the absence of an *ortho*-dihydroxyl pattern in B-ring, while **B**, **C** and **E** gave the orange fluorescence with NA characteristic of a catechol system in B-ring. The last was confirmed by the UV shifts on addition of the diagnostic reagents [27- 30].

It was clear from the UV spectra, that **A-F** belonged to flavonols with the sugar residues (compounds **B-F**) attached to C-3 [27, 29]. Upon the acid hydrolysis **B**, **C** and **D** released the aglycones - querectin (for **B** and **C**) or kaempferol (**D**), along with glucose (**C** and **D**) or arabinose (**B**). Partial hydrolysis of **E** and **F** gave the secondary heterosides, chromatografically identical with **C** or **D**, respectively. The total hydrolysis of **E** and **F** led to quercetin (**E**) or kaempferol (**F**) as well as glucoses and rhamnoses, thus confirming the presence of two sugar moieties in both compounds.

The aglycone region in ^{1}H NMR spectra showed signals characteristic for quercetin (**C**, **E**) or kaempferol (**D**, **F**). In the sugar area there were seen signals of one (**C** and **D**) or two anomeric protons (**E** and **F**), which supported the former findings. The anomeric protons of glucoses resonated at 5.44–5.46 ppm (**D** and **C**) or 5.20–5.31ppm (**F** and **E**), in the area typical for 3-*O*- glycosides, with the coupling constants of 7.7-8.0 Hz confirming β-glucosidation and a pyranose form of all of them. The second anomeric signals at 4.37 (**E**) or 4.50 ppm (**F**) were assigned to the α-linked terminal rhamnoses. H-6''' resonated at 0.96 ppm (J = 6.2 Hz) for **E** or at 1.00 ppm (d, J=5.0 Hz, H-6) for **F**. Their positions as well as J values, were indicative of the terminal rhamnose linked to the glucose *via* 1→6 linkage [27, 29, 31].

All those findings were confirmed by ^{13}C NMR analysis. In the ^{13}C NMR spectra of (**C-F)** the upfield position of the C-3 signals (by 2,3-2,7 ppm) as well as the downfield shifts of the *ortho* (C-2 and C-4) and *para* (C-10) carbon signals, in relation to the appropriate signals of their aglycones (quercetin for **C** and **E** and kaempferol for **D** and **F**), proved the sugar linkage to C-3; while the anomeric carbons appeared in the region typical for *O*-glycosides. Moreover, the downfiled shifts of C-6''' (**E** and **F**), of about 6 ppm, confirmed the (1→6) linkage between rhamnose and glucose [29, 32-34].

The obtained results were in agreement with the literature data [31, 32, 35] for: quercetin 3-*O*-β-glucopyranoside [**C**], kaempferol 3-*O*-β-glucopyranoside [**D**], quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside [**E**] and kaempferol 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside [**F**]. The identification of **B** was based on the results of co-chromatografic studies of primary compound and the products of its acid degradation, as well as on the UV spectra.

CONCLUSION

In our studies we have isolated six flavonoid compounds from the *Tussilago farfara* leaves, which were subsequently identified as kaempferol and its derivatives: 3-*O*-β-glucopyranoside and 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside, and

quercetin derivatives: 3-*O*-*β*-arabinopyranoside, 3-*O*-*β*-glucopyranoside and 3-*O*-*α*-rhamnopyranosyl(1→6)-β-glucopyranoside. The composition of flavonoid fractions from the coltsfoot flowers and leaves is similar – in both cases glycosides of the most common flavonol – quercetin and kaempferol were present (tab. 1). As it may be seen from the comparison of the literature data with our results, the coltsfood flowers were more abundant – they contained some extra compounds which were absent in the leaf extracts: quercetin, quercetin 3-*O*-*β*-galactopyranoside, quercetin 4'-*O*-*β*-glucopyranoside, and also kaempferol 3-*O*-*β*-arabinopyranoside. In addition to the abovementioned flavonoids, we have also detected the presence of three simple phenolic acids: *p*-hydroxybenzoic, *cis-, trans- p*-coumaric, and *cis-, trans-* chlorogenic. To the best of our knowledge this is the first report on the presence of the fore mentioned phenolics in the leaves of *Tussilago farfara* L.

All phenolics identified in the studied coltsfoof leaves may contribute to the pharmacological effects of that herb. Flavonoids are well known antioxidants and inhibitors of the arachidonic acid cascade enzymes (LOX, COX, PAL_2) [36]. The last activity has been proven i.e. for astragalin and isoquercitin [37], while Park et al. [38] have shown that rutin inhibits the expression of pro-inflammatory factors: TNF-*α*, IL-1*β*, IL-6 and IL-8. What is more, rutin, especially in combination with vitamin C, possesses anti-allergic activity [39]. Chlorogenic acid, apart from many other biological effects, is recognized as a natural agent lowering postprandial hyperglycemia [40].

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ZWIĄZKI FENOLOWE W LIŚCIACH PODBIAŁU

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S t r e s z c z e n i e

Liście podbiału (*Farfarae folium*) są stosowane w medycynie europejskiej w chorobach dróg oddechowych, w kaszlu, w stanach zapalnych i skurczowych oskrzeli. W medycynie chińskiej są wykorzystywane jedynie kwiaty podbiału (*Farfarae flos*), dlatego większość danych literaturowych dotyczy składu chemicznego kwiatów. Z liści podbiału wyizolowaliśmy i zidentyfikowaliśmy (UV, ^{1}H i ^{13}C NMR, hydroliza kwasowa i enzymatyczna) 6 związków flawonoidowych: kemferol i jego glikozydy: 3-*O*-β-glukopiranozyd i 3-*O*-α-ramnopiranozylo(1→6)-β-glukopiranozyd, oraz glikozydy kwercetyny: 3-*O*-β-arabinopiranozyd, 3-*O*-β-glukopiranozyd i 3-*O*-α-ramnopiranozylo(1→6)-β-glukopiranozyd. Ponadto w surowcu stwierdzono obecność trzech kwasów fenolowych.

Słowa kluczowe: *Tussilago farfara, liście, flawonoidy, kwasy fenolowe*