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Original Article

ANTIPROLIFERATIVE EFFECT OF FLOWER EXTRACTS OF SPILANTHES PANICULATA ON HEPATIC CARCINOMA CELLS

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ABSTRACT

Objective: The development and evaluation of new antiproliferative drugs obtained from natural resources has gained its importance because of their less cytotoxic properties. There is no such report regarding antiproliferative effect of *Spilanthes paniculata Linn*. flower. Therefore, the present study was undertaken to evaluate the antiproliferative effect of *Spilanthes paniculata Linn*. flower.

Methods: In order to achieve this goal, the dried flowers were extracted in petroleum ether, ethyl acetate and ethanol. All these three extracts of varying concentrations were subjected to further evaluation of antiproliferative action on human hepatoma cell line (Huh-7 cells). In order to understand the mechanism of antiproliferative effect of these three extracts, various studies like caspase-3 enzyme assay, DNA transillumination assay and receptor tyrosine kinase profiling were performed. Separately, we estimated total flavonoid and phenolic contents and *in vitro* free radical scavenging properties of these extracts.

Results: The results indicated that both ethyl acetate and ethanol extracts possessed antiproliferative effect on Huh-7 cells because of their induction of caspase-3 enzymes and inhibition of phosphorylation of various tyrosine kinases. It was observed that during the transillumination assay of the ethyl acetate and ethanol extracts, DNA of Huh-7 cells were also degraded. It was also found that these two extracts possessed potent antiproliferative effect on Huh-7 cells due to the presence of rich amount of phenols and flavonoids.

Conclusion: Based on our data, both ethyl acetate and ethanol extracts might be beneficial for the future development of antiproliferative therapeutics in drug design perspective.

Keywords: *Spilanthes paniculata* flowers, Antiproliferative effect, Huh-7 cells, Caspase-3, DNA transillumination, Tyrosine kinase.

INTRODUCTION

From the ancient periods, human beings have believed on natural products, which are the common source of therapeutics. Egypt, China and India are the common countries where natural products have been used in healthcare system [1]. World Health Organization published a survey report on primary health care system where they explained about 80% of world population has faith on traditional medicines [2].Currently, 119 chemicals obtained from 90 plant species are considered as important therapeutics in different countries [3].

Spilanthes paniculata Linn. (SP) belongs to the family Asteraceae,also known as toothache plant, akalkada (in Sanskrit) and akarkara (in Hindi). The traditional uses of this plant are to relief toothache, rheumatic fever. Leaves are mainly used against various skin diseases whereas the extract of root is used as purgative [4]. The active constituent of SP is N-isobutylamide including spilanthol which is mainly responsible for above mentioned pharmacological actions [5]. Thomas (2011) observed that the different fractions of flower head of SP were active against gram positive and gram negative bacteria [6]. Ali et al., (2012) evaluated the hepatoprotective effect of SP flower on paracetamol induced hepatotoxicity due to presence of rich sources of flavonoid and phenolic contents [7].

Thus, taking into consideration of above mentioned observations, the present study was conducted to evaluate the antiproliferative effect of SP flower on hepatic carcinoma cells (Huh-7 cells) [8,9]. In order to achieve the desired goal, extraction of SP dried flowers (shade drying) was done by using petroleum ether (60-80°C, PE), ethyl acetate (EA) and ethanol (ET). All these extracts were subjected to further screen for antiproliferative action on Huh-7 cells at different concentrations from 0.5 to 100 µg/ml, followed by apoptotic activity induced by caspase-3 enzymes. Receptor tyrosine kinase (RTK) profiling and DNA transillumination assay were also performed to explain the actual mechanism of antiproliferative effects of these extracts. Separately, we also estimated total phenolic and flavonoid contents and free radical scavenging assays of those extracts. We hypothesized for the first time that both EA and ET extract of SP flower had good antiproliferative effect on Huh-7 cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and caspase-3 enzyme assay kit were purchased from Invitrogen Bio Services Pvt. Ltd., India. Petroleum ether (PE), ethyl acetate (EA), and ethanol (ET) were purchased from Merck Pvt. Ltd., India. Dimethyl sulfoxide (DMSO, ACS grade), ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$, 99% purity) and trypsin ethylenediaminetetra acetic acid (trypsin-EDTA) were obtained from S. D. Fine Chemicals, India. Huh-7 cell lines were purchased from ATCC, Manassas, VA, USA. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents of analytical grades were purchased from Himedia Pvt. Ltd., India.

Preparation of Extracts

The flowers of SP were collected from costal area of West Bengal in the month of August-September, authenticated by Botanical Survey of India, Howrah, India with a voucher specimen No. 142/BSI/2013 and were deposited for future reference. At first, the dried flowers were crushed by using a mixer grinder (Bajaj Appliances Pvt. Ltd., India) and powered accordingly. About 150 g of powder was taken in a Soxhlet apparatus and the extraction was carried out using PE, EA and ET as an extracting solvents separately for consecutive three days. Finally, the solvent was evaporated through rotary vacuum evaporator and yields of extracts in different extracting solvent were calculated. The yields were 10, 12 and 14.5% for PE, EA and ET extracts, respectively.

Preparation of Huh-7 cells

Huh-7 cells were cultured in DMEM, which was supplemented with 10% (v/v) foetal bovine serum and 100 IU/ml penicillin/ streptomycin. Cells were maintained in humid condition of 5% CO_2 environment at 37°C.

MTT viability assay

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] viability assay was performed as per the protocol reported earlier in the literature [10]. Briefly, Huh-7 cells were seeded in 96-wells plate (5.0×10^3cells/well) and kept for 24 h. Then, 200 µl of varying concentrations of extracts (0.5 to 100 µg/ml) was added to each well and the samples were incubated in humid condition of CO_2 at 37°C. After 72 h, 50 µl of MTT solution (1 mg/ml) was added to each well and incubated for another 4 h. Then, the media and excess MTT were removed and 150 µl of DMSO was also added. The absorbance was recorded at 570 nm using the Infinite M200 microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). Samples containing media with and without cells were also analyzed and labeled as 'control' and 'blank', respectively. All the experiments were performed in triplicates. The final organic solvent concentration was 0.3% (v/v). Cell survival (% of control) was calculated relative to untreated control cells.

Caspase-3 enzyme assay

Caspase-3 enzyme assay was performed on Huh-7 cells to measure the apoptotic activity that was described previously [11]. In brief, $2x10^4$ cells/well was seeded in 96-wells plate and divided into control (DMSO, 1%, 100 µl) and varying concentration of test groups (0.5, 1.0, 10.0, 25.0, 50.0 and 100.0 µg/ml) at 37°C. After 24 h, 100 µl of Caspase-3 reagent was added to the mixture and further incubated for another 2 h. Relative Luminescence Unit (RLU) was recorded using the Infinite M200 microplate reader (Tecan Group Ltd., Mannedorf, Switzerland).

DNA transillumination assay

DNA transillumination assay was performed as per the method reported earlier [12].Huh-7 cells ($1x10^6$cells/ml) were treated with EA and ET extracts at 10 µg/ml concentration for 24 h. Next day, the treated cells were re-suspended in 500 µl of lysis buffer containing (50 mM Tris-HCl buffer of pH 8.0, 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate), 10 µg/ml of protenase-K was added and kept at 37°C for 10 min. After incubation, the phenol-chloroform procedure was conducted and kept it for overnight at −20°C. Next day, DNA was precipitated after centrifugation, washed with 70% (v/v) ethanol, dried, evaporated at room temperature. Then, it was dissolved in Tris-EDTA buffer solution and kept at 4ºC. The isolated DNA samples were electrophoresed at 20V in 1% (w/v) agarose gel for 2 h. DNA fragmentation was observed in UV transilluminator (Biorad, USA).

Receptor tyrosine kinase (RTK) profiling

RTK assay was performed as per the protocol reported earlier [13]. Huh-7 cell were treated with 10 µg/ml of extracts (PE, EA and ET) and a control consisting of 0.4% (v/v) of DMSO in 15 ml petri dishes and was kept for 24 h. The cells were scrapped and lysed using lysis buffer and the assay was performed by using the Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, USA).

Determination of total phenolic contents (TPC) in extracts

Total phenolic content (TPC) in extracts was determined as per the method reported earlier [14]. The reaction mixture consisted of 0.5 ml of 0.1% (w/v) extract in methanol, 2.5 ml of 10% (v/v) Folin-Ciocalteu reagent in water and 2.5 ml of 7.5% (w/v) of sodium bicarbonate in water. Blank solution was prepared in similar procedure where extracts were absent. Then, all samples were incubated at 37°C for 1 h and absorbance was measured spectrophotometrically at 765 nm. The same procedure was also adopted for standard gallic acid (GA) and TPC was determined from the calibration curve constructed and expressed in terms of GA equivalent (mg of GA/g of extract).

Determination of total flavonoid contents (TFC) in extracts

Total flavonoid contents (TFC) in extracts were determined as per the method described previously [15]. The test sample contained 1 ml of 0.1% (w/v) extract and 1 ml of 2% (w/v) aluminum chloride in methanol. Control samples were also prepared in the similar procedure where extracts were absent.

samples were kept at 37°C for 1 h and absorbance was measured spectrophotometrically at 415 nm. Same procedure was followed for standard rutin (RU) and TFC was determined from the calibration curve constructed and expressed in terms of RU equivalent (mg of RU/g of extract).

Free radical scavenging assays

Various free radical scavenging assays had also been performed during our experiment to check out the antioxidant capacities of EA and ET extracts. Free radical scavenging assays like 1,1-diphenyl-2-picrylhydrazine (DPPH), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) assays were performed which had been described previously [16, 17].

RESULTS

MTT viability assay

It was observed that PE extract had lowest action on anti proliferative effect as shown in fig. 1, whereas EA and ET extracts showed the said action above a critical concentration of 1.0 µg/ml. It was also observed that ET extract showed 4 to 90% inhibition on Huh-7 cells, whereas EA extract showed 20 to 90% inhibition. The calculated EC_{50} values of PE, EA and ET extracts were 43.16, 18.33 and 4.19 µg/ml, respectively. Statistically significant differences were observed between PE and EA extracts (paired t-test, $P<0.05$ at 25, 50 and 100 µg/ml concentrations) and EA and ET extracts (paired t-test, $P<0.05$ at and above 10.0 µg/ml concentration).

Caspase-3 enzyme assay

The results indicated that all these extracts (PE, EA and ET) produced dose dependent increase in apoptosis on Huh-7 cells with respect to control group as shown in fig. 2A to 2C. It is obvious form the graph that ET extract showed two and three times higher apoptotic action than EA and PE extracts, respectively. Statistically significance differences were observed between control and test groups for these extracts (One-way ANOVA followed by Bonferroni multiple comparison test, $P<0.05$) at all concentrations with respect to control.

DNA transillumination assay

The Huh-7 cells were treated with both EA and ET extracts at a dose of 10 µg/ml concentration for 24 h. The DNA isolated from Huh-7 cells showed intact band in control whereas degraded DNA was found in the form of ladders or smear in case of EA and ET treated cells (fig. 3).

Receptor tyrosine kinase (RTK) profiling

In order to understand the targets for antiproliferative property, Huh-7 cells were treated with three extracts at a concentration of 10 µg/ml for 24 h and subjected to phospho-analysis among various targets (Supplementary Data Sheet table 1 and table 2). Phosphorylation of EGFR and MET were completely suppressed when cells were treated with 10 µg/ml of ET extract. Other targets like ROR, VGFR3 and RON exhibited pronounced inhibition (Table 1). It was obvious from table 1 that EA extract showed moderate effect on inhibition, whereas PE extract stated no suppression on phosphorylation.

Determination of total phenolic contents (TPC) in extracts

The phenol content was found to be 41 and 30 mg/g for ET and EA extracts, respectively, whereas only 11 mg/g was found in case of PE extract (Table 2). Total Phenolic content in the extract depends on the type of solvent used for extraction. Because of high polarity, it was obvious that more phenol extracted in polar solvent and showed higher concentration in EA and ET extracts [18].

Determination of total flavonoid contents (TFC) in extracts

The flavonoid content was found to be 48 and 25 mg/g for ET and EA extracts, respectively, whereas only 10 mg/g was found in case of PE extract (Table 2).

Because of high polarity, it was obvious that more flavonoid extracted in polar solvent and showed higher concentration in EA and ET extracts [19].

Free radical scavenging assays

We performed DPPH, H_2O_2 and. OH assays to measure scavenging properties of extract. According to fig. 4, it was observed that both extract had % inhibition effects in a dose dependent manner. However, ET extract showed higher inhibitory effect than EA extract. ET extract produced from 5 to 80% inhibition at concentrations from 1 to 100 µg/ml whereas EA extract had only 5 to 20% inhibition in the same concentration (fig. 4A to 4C). PE extract had no such effect (data not shown).

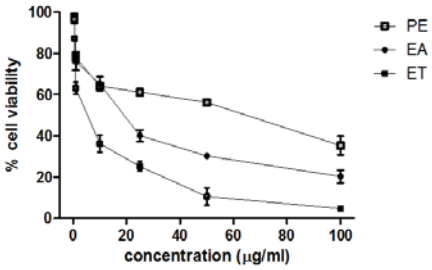
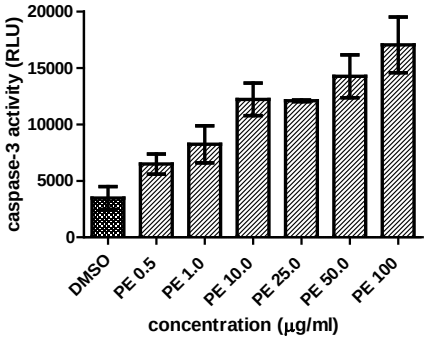
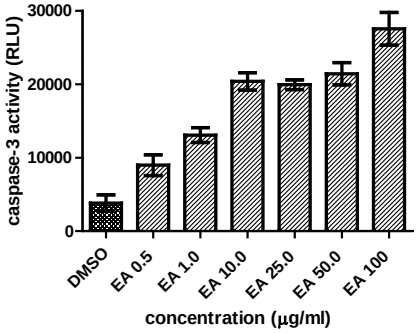


Fig. 1: Percentage viability of Huh-7 cells after treatment with PE, EA and ET extracts of SP flower (0.5, 1, 10, 25, 50 and 100 µg/ml) for 72 h prior to MTT assay to determine potential anticancer effect. The calculated EC_{50} values of PE, EA and ET extracts were 43.16, 18.33 and 4.19 µg/ml, respectively. Statistically significant differences were observed between PE and EA extracts (paired t-test, P<0.05 at 25, 50 and 100 µg/ml concentrations) and EA and ET extracts (paired t-test, P<0.05 at and above 10.0 µg/ml concentrations)



(A)



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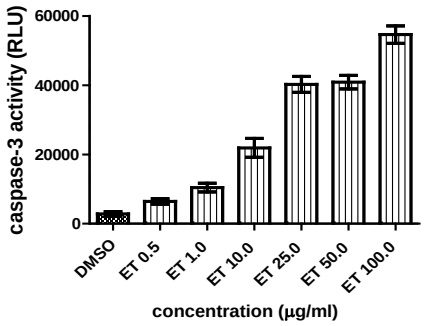


(C)

Fig. 2: Caspase-3 activities was expressed in Relative Luminescence Units (RLU) for characterizing the anticancer property of varying concentrations (µg/ml) of (a) PE, (b) EA and (c) ET extracts of SP flower on Huh-7 cells. Statistically significance differences were observed between normal control and test groups (One-way ANOVA followed by Bonferroni multiple comparison test, P<0.05) at all concentrations

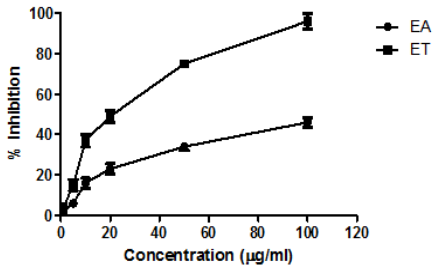
DISCUSSION

Treatment of cancer in epithelial region of human body remains disappointing until twenty first century. The usage of synthetic antiproliferative chemotherapeutic agents has declined due to its potential toxicity to human body. Moreover, the compounds obtained from natural origin are safe, less toxic in nature. Therefore, it is necessary to investigate potential therapeutic agents for the clinical benefits among cancerous patients. Recent investigation suggested that SP flowers are rich in phenols and flavonoids [7] which might be contributed for its antiproliferative properties [8, 9]. On this contrary, our group investigated the antiproliferative effect of SP flower using various extracts. Initially, SP flowers were extracted with PE, EA and ET solvents separately and these three extracts were subjected to further pharmacological screening.

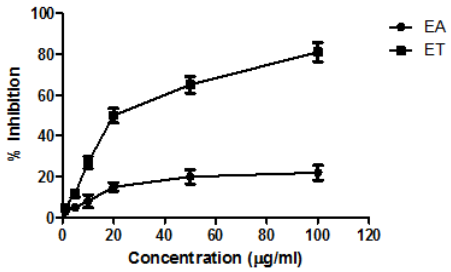
Antiproliferative activity was performed on Huh-7 cells, where MTT was used as detecting reagent [10, 12]. We found that both EA and ET extracts of SP flower possessed antiproliferative action at lower concentration (1 µg/ml) whereas PE extract had similar effect on higher concentrations. Both EA and ET extracts had lower EC_{50} values as shown in fig. 1. However, this action was less prominent for PE extract in the same experiment. This was the primary indication of antiproliferative property of SP flower.

Characterization of this property of these extracts was analyzed through caspase-3 activity, expressed in RLU and compared against DMSO-containing vehicle control. This assay is an indirect indication for the measurement of apoptotic activity in treated cells as a phenotype of anti-cancer potential. All the extracts showed dose dependent increase in caspase-3 enzymes. ET extract showed highest induction on caspase-3 enzyme (two and three times higher than EA and PE extracts, respectively) whereas EA extract expressed moderate effect on caspase-3 (fig. 2). Caspase-3 is the key enzyme responsible for programmed cell death through cleavage and inactivation of structural constituents and fragmentation of genomic DNA of cancerous cells [20].Both EA and ET extracts showed increase in caspase-3 enzyme level which signified their antiproliferative potential.

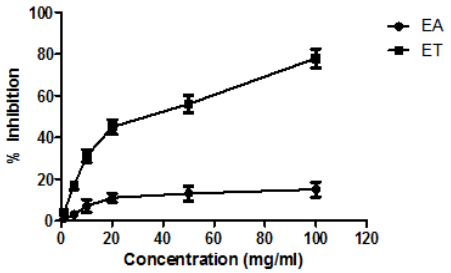
Fig. 3: Huh-7 cells (1x10^6 cells/ml) were treated with EA & ET at 10 µg/ml for 24 h. The isolated DNA samples were electrophoresed for 2 h at 20V in 1% agarose gel and stained with ethidium bromide. We found intact DNA for control group (A) whereas degraded DNA as smear or broken ladder in EA (B) and ET (C) groups



(A)



(B)



(C)

Fig. 4: Free radical scavenging assays [(A) DPPH, (B) H_2O_2 and (C) hydroxyl radical] of EA and ET extracts from 1 to 100 µg/ml concentration

Table 1: Effect of fold change in RTK phosphorylation with respect to control when treated with 10 µg/ml on the treatment target for 24 h. Registered treated targets are displayed as 2-fold change. ET extract expressed highest inhibitory effect on phosphorylation of any targets than other extracts

RTK targets	PE	EA	ET
EGFR	2.99	0.77	-0.01
MET	3.16	0.62	-0.08
ROR2	2.07	1.02	0.34
VEGFR3	1.95	0.86	0.25
RON	1.85	1.34	0.57

Table 2: TPC in terms of GA equivalent and TFC in terms of RU equivalent were measured in both three extracts. Values were represented as mean ± SD (n=3). Both TPC and TFC were higher for ET extract than PE and EA extracts.

Extracts	PE	EA	ET
TPC (mg of GA/g of extract)	11.85±1.74	30.71±1.93	41.56±2.41
TFC (mg of RU/g of extract)	10.28±0.52	25.83±2.23	47.96±2.89

Now, the question arose whether these two extracts (EA and ET) produced any antiproliferative action on hepatoma cells other than the mechanism of caspase-3. In order to get the answer, we performed DNA transllumination assay where we observed the degradation of DNA in the test groups (fig. 3). Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes, are eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles [20,21]. Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer, resulting in a loss of membrane integrity, swelling and disruption of the cells. Further evidence in support of the apoptogenic activity of EA and ET were obtained from the gel patterns in electrophoresis on Huh-7 cells. Treated cells showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator. It is also necessary to know the alternative target of antiproliferative properties of these two extracts. Recently, inhibition of RTK enzymes is another important target for the development of antiproliferative drugs. It is

assumed that these enzymes are helpful for phosphorylating the subsequent protein and initiating the signaling cascade for cancer development [22], whose inhibition resulted in good antiproliferative potential. Based on the previous RTK profiling assay, ET extract inhibited a number of targets like EGFR, MET, ROR2, VEGFR3 and RON (Table 1) whereas EA extract has moderate effect on inhibition.

Previous investigation by Ren et al., (2003) [8] and Abubakar et al., (2012) [9] revealed that both phenolics and flavonoids are mainly responsible for antiproliferative action. Therefore, it is necessary to know the amount of phenolics and flavonoids present in flower head. Both TPC and TFC were measured biochemically where we found that the content of phenolics and flavonoids in ET extract was higher than EA and PE extracts. This is the main reason behind the highest antiproliferative activity of ET extract.

Free radicals and reactive oxygen species have important role in cancer homeostasis and development [23]. Hence, it is necessary to know the scavenging properties of both EA and ET extracts to find out the possible mechanism of antiproliferative effect. Previous investigation suggested that flavonoids from natural origin have promising effects on free radical scavenging activity [24]. Various assays revealed that ET extract had promising effect on radical scavenging as it contains higher amount of flavonoids than EA extract.

CONCLUSION

Recent investigation suggested that both phenols and flavonoids from natural origin have good antiproliferative activity due to free radical scavenging effect [24]. ET extract of SP flower gave better antiproliferative effect due to the presence of higher amount of these contents. The antiproliferative action of ET extract may be due to other mechanisms like induction the caspase-3 enzyme, inhibition of tyrosine kinase enzyme and degradation of cellular DNA. EA extract had similar effect but it was slightly lower than ET extract. Our final conclusion is that both EA and ET extracts had good antiproliferative effect on Huh-7 cells and might be better alternative for future antiproliferative drug screening.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ABBREVIATIONS

(SP), *Spilanthes paniculata*; (PE), Petroleum ether; (EA), Ethyl acetate; (ET), Ethanol; (Huh-7 cells), Human hepatic carcinoma cells; (DMEM), Dulbecco's modified Eagle's medium; (DMSO), Dimethyl sulfoxide; ($Na_2EDTA.2H_2O$), ethylenediaminetetraacetic acid disodium salt dihydrate; (trypsin-EDTA), trypsin ethylenediaminetetraacetic acid; (MTT), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (RLU), Relative Luminescence Unit; (RTK), Receptor tyrosine kinase; (TPC), total phenolic contents; (TFC), total flavonoid contents; (GA), gallic acid; (RU), rutin;

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