NUTRITION AND CANCER
An International Journal

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Title

Salvia fruticosa, *Salvia officinalis* **and rosmarinic acid induce apoptosis and inhibit**

proliferation of Human Colorectal cell lines: the role in MAPK/ERK pathway

Cristina P.R. Xavier[1], Cristovao F. Lima[1], Manuel Fernandes-Ferreira[2] and Cristina Pereira-Wilson[1]

[1]CBMA – Molecular and Environmental Biology Centre/Department of Biology, University of Minho, 4710-057 Braga, Portugal
[2]CMPBP – Centre of Molecular Physiology and Biotechnology of Plants/Department of Biology, University of Minho, 4710-057 Braga, Portugal

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1　**ABSTRACT**

2　　　　Epidemiologic studies have shown that nutrition is a key factor in modulating

3　sporadic colorectal carcinoma (CRC) risk. Aromatic plants of the genus *Salvia* (sage)

4　have been attributed many medicinal properties, which include anticancer activity. In

5　the present study, the antiproliferative and pro-apoptotic effects of water extracts of

6　*Salvia fruticosa* (SF) and *Salvia officinalis* (SO) and of their main phenolic compound

7　rosmarinic acid (RA) were evaluated in two human colon carcinoma-derived cell lines,

8　HCT15 and CO115, which have different mutations in the MAPK/ERK and PI3K/Akt

9　signalling pathways. These pathways are commonly altered in CRC leading to increased

10　proliferation and inhibition of apoptosis. Our results show that SF, SO and RA induce

11　apoptosis in both cell lines, whereas cell proliferation was inhibited by the two sage

12　extracts only in HCT15. SO, SF and RA inhibited ERK phosphorylation in HCT15 and

13　had no effects on Akt phosphorylation in CO115 cells. The activity of sage extracts

14　seems to be due, at least in part, to the inhibition of MAPK/ERK pathway.

15

16　**Introduction**

17　　　　Cancer is an important health problem and one of the most common forms is

18　colorectal carcinoma (CRC). Phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-

19　activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling

20　pathways play critical roles in cell proliferation and survival and are frequently

21　activated in CRC (1-3). Deregulation of these pathways is also thought to determine

22　response to treatment (4). Mutations of KRAS and BRAF in sporadic CRC [70-80% of

23　total cases (5)] are alternative, where the former constitutively activates both

24　MAPK/ERK and PI3K/Akt pathways and the latter activates MAPK/ERK pathway (3,

25　4, 6-8). As presented by Schubbert *et al*. (9), mutations in CRC of either KRAS or

26   BRAF genes occur in 32% and 14% of cases, respectively. Studies have also shown that

27   CRC is frequently associated with mutations in genes that encode for PI3K, PI3KCA,

28   and PTEN (an endogenous inhibitor of PI3K activity), resulting in an overexpression of

29   Akt (10-13). Considering the high incidence of CRC, inhibitors of these pathways are

30   actively being searched for use in the control of cancer progression (14-16).

31   Epidemiologic studies have shown that western type diets, poor in vegetables

32   and fruits, are risk factors known associated with CRC, suggesting that nutritional

33   factors may also be preventive and also helpful in the control of cancer (17-19). In fact

34   green and black tea consumption has been shown to be effective in the initiation,

35   promotion and progression stages of carcinogenesis, although effects on colon cancer

36   are inconclusive (20). Plants of the genus *Salvia* (sage) such as *Salvia miltiorrhiza* and

37   *Salvia menthaefolia* have also been suggested to have anticancer properties based on

38   antiproliferative activity on tumor cells (21, 22). In addition, reactive oxygen species

39   (ROS) have been reported to play a role in signalling transduction enhancing

40   proliferation and survival of cancer cells. Antioxidant phytochemicals through their

41   ROS scavenging activity, may suppress altered redox-sensitive signalling events in

42   cancer (23, 24).

43   *Salvia fruticosa* (SF) and *Salvia officinalis* (SO), poorly studied with regard to

44   their anticancer activity, are mediterranean medicinal and aromatic plants which contain

45   rosmarinic acid (RA; Fig. 1) as major phenolic compound in their water extracts. RA

46   constitutes about 58% of all phenolic compounds present in SF water extract and 70%

47   in SO water extract (25, 26). This phenolic compound has high antioxidant and anti-

48   inflammatory activities (22, 27), but little is known about its effects on cancer cells and

49   especially on CRC.

50   In the present study, we report on the antiproliferative and pro-apoptotic effects

51   of two *Salvia* water extracts, SF and SO, and their major phenolic compound, RA, in

52   two human colon cancer-derived cell lines, HCT15 and CO115, through effects on the

53   MAPK/ERK and PI3K/Akt pathways and caspase mediated apoptosis. These two cell

54   lines possess different activating mutations in these two pathways: HCT15 has a KRAS

55   (G13D) mutation (28) whereas CO115 has a BRAF (V599E) mutation (29).

56   In view of these genetic differences we further speculate on the mechanisms

57   behind the antiproliferative and pro-apoptotic effects of sage extracts and RA and the

58   involvement of PI3K/Akt and MAPK/ERK signalling pathways in these effects.

59

60   **Material and Methods**

61

62   **Reagents and Plant Extracts**

63   All reagents and chemicals used were of analytical grade. Wortmannin (W),

64   rosmarinic acid (RA) and staurosporine were purchased from Sigma-Aldrich (St. Louis,

65   MO, USA) and PD-98059 (PD) was from Calbiochem (San Diego, CA, USA). The

66   primary antibodies anti-phospho-Akt (Ser473), anti-Akt total, anti-phospho-PTEN

67   (Ser380/Thr382/383), anti-PTEN total, anti-p44/42 MAPK total and anti-cleaved

68   caspase-9 (Asp315) were purchased from Cell Signaling (Danvers, MA, USA), the anti-

69   phospho-ERK and caspase-3 (H-277) were from Santa Cruz Biotechnology, Inc. (Santa

70   Cruz, CA, USA) and the anti-β-actin from Sigma-Aldrich. The secondary antibodies

71   HRP donkey anti-rabbit and sheep anti-mouse were from GE Healthcare (Bucks, UK).

72   The water extracts of *Salvia fruticosa* and *Salvia officinalis* were prepared as

73   previously described by Lima *et al.* (30), by pouring boiling water onto the dried plant

74   material (at ratio of 150ml of water to each 2g of plant) and allowing to steep for 5min.

75   After filtering, the water extract was lyophilized to dryness. The extracts of both sages

76   were made using batches of the plants which composition, in terms of phenolics

77   compounds, have already been published (25, 26). In brief, SF water extract contain as

78   major phenolic compound rosmarinic acid (RA; 71.5µg/ml), 6-hydroxyluteolin-7-

79   glucoside (22.7µg/ml), a not identified flavone heteroside (28.6µg/ml) and the

80   remaining phenolic compounds representing 0.8µg/ml. SO water extract contain as

81   major phenolic compounds RA (52.0µg/ml), luteolin-7-glucoside (19.7µg/ml) and the

82   remaining phenolic compounds representing 2.7µg/ml.

83   Stocks solutions of PD and W were made in dimethyl sulfoxide (DMSO) and

84   aliquots were kept at -20ºC. Therefore, DMSO (0.5%) was included in cell culture for

85   the other conditions (controls and extracts/RA) to exclude any possible DMSO effect.

86

Cell culture

87

88   HCT15 and CO115 human colon carcinoma-derived cell lines were a gift from

89   Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained in

90   culture at 37ºC in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-

91   Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic-

92   antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard,

93   Cambrex, Verviers, Belgium). Cells were seeded onto six well plates at a density of

94   0.75 $x10^5$ (HCT15) and 1.0 $x10^5$ (CO115) cells/well. Incubations with different

95   concentrations of sage extracts and RA were performed in serum free medium for 48h

96   to analyze BrdU incorporation and TUNEL positive cells, and for 24h for western blot

97   analysis.

98

Assessment of proliferation by BrdU incorporation

99

100   Preliminary experiments using the MTT assay were performed in order to

101   choose concentrations of SF and SO extracts that inhibited around 50% cell

102   proliferation without cytotoxic effects. RA was tested in similar concentrations to the

103   ones found in the extracts at the concentrations used and also did not induce cytotoxic

104   effect. After 45h of treatment with sage extracts or RA at different concentrations,

105   bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the culture medium in order to

106   give a final concentration of 10μM, and then incubated for another 3h. Both adherent

107   and non-adherent cells were collected from each sample, fixed with 4%

108   paraformaldehyde for 15min at room temperature and then attached into a polylysine

109   treated slide using a Shandon Cytospin (Thermo Fisher Scientific Inc, Waltham MA,

110   USA). Cells were incubated with HCl 2M for 20min, washed in PBS containing 0.5%

111   Tween-20 and 0.05% BSA (TPBS-B) and then incubated with monoclonal mouse anti-

112   BrdU antibody (DakoCytomation, Glostrup, Denmark) for 1h at room temperature.

113   After washing in TPBS-B, cells were incubated with anti-mouse IgG FITC-conjugated

114   secondary antibody (Sigma-Aldrich) for 1h at room temperature, washed again and then

115   incubated with Hoechst for nuclei staining. The percentage of proliferating cells was

116   calculated as the ratio between BrdU positive cells and total number of cells (nuclei

117   staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent

118   microscope. Results are presented as mean ± SEM of at least three independent

119   experiments.

120

121   **Assessment of apoptosis by TUNEL assay**

122   Cells treated as above for 48 h were collected (both floating and attached cells)

123   and fixed with 4% paraformaldehyde for 15min at room temperature and then attached

124   into a polylysine treated slide using a Shandon Cytospin. Cells were washed in PBS and

125   permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL

126   (TdT mediated dUTP Nick End Labelling) assay was performed using a kit from Roche

127   (Mannheim, Germany), following the manufacture's instructions. Cells were incubated

128   with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from

129   the ratio between TUNEL positive cells and total number of cells (nuclei staining with

130   Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope.

131   Results are presented as mean ± SEM of at least three independent experiments.

132

133   **Protein extraction and western blotting**

134   After 24h of treatment with sage extracts or RA at the highest concentration used

135   in the BrdU and TUNEL assay, cells were washed with PBS and lysed for 15min at 4ºC

136   with ice cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM

137   EDTA), supplemented with 20mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF),

138   20mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche). Protein concentration was

139   quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules,

140   CA, USA) and BSA as a protein standard. Twenty micrograms of protein for each

141   sample were separated by SDS gel electrophoresis and then electroblotted to a Hybond-

142   P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in

143   TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA,

144   incubated with the primary antibody and followed by the secondary antibody

145   conjugated with IgG horseradish peroxidase. Membranes were washed 3 times with

146   TPBS between the different incubations. Immunoreactive bands were detected using the

147   Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence

148   detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity

149   was quantified using the Quantity One software from Bio-Rad. β-actin was used as a

150   loading control. Results are presented as mean ± SEM of at least three independent

151   experiments.

152

Statistical analysis

154   One-way ANOVA followed by the Student-Newman-Keuls test was used to

155   perform statistical analysis for BrdU, TUNEL and western blot data. GraphPad Prism

156   4.0 software (San Diego, CA, USA) was used and *P*-values ≤0.05 were considered

157   statistically significant.

158

Results

160

Effects on cell proliferation

162   To test the effects of SF, SO and RA on cell proliferation of human colon cancer

163   cells, two different colon carcinoma-derived cell lines, HCT15 and CO115, were used.

164   Based on preliminary experiments using the MTT assay (data not shown), where

165   cells were incubated with several concentrations of sage extracts for 48h, concentrations

166   of each extract that were not cytotoxic and inhibited cell proliferation around 50% were

167   chosen for the subsequent studies. Since RA is the main phenolic compound of these

168   extracts, we also tested RA in similar concentrations to the ones found in the extracts

169   under our experimental conditions.

170   The effects of sage extracts and RA on cell proliferation of both cell lines were

171   tested using the BrdU incorporation assay. As shown in Fig. 2A, a significant inhibition

172   of HCT15 cell proliferation by both SF and SO was observed at all concentrations

173   tested. Levels of BrdU incorporation significantly decreased from 26.2% in the control

174   to 4.7% in HCT15 cells treated with 50μg/ml of SF and SO extracts. In CO115 cells, SF

175   and SO did not inhibit significantly cell proliferation (Fig. 2B). No significant inhibition

176   of cell proliferation was observed in both cell lines treated with RA (Fig. 2). Comparing

177   the effects of sage extracts in the two cell lines, we observed that SF extract was

178   somewhat more active than SO and HCT15 cells were more sensitive to the sage

179   extracts.

180

181   **Effects on apoptosis**

182   The ability of SF, SO and RA to induce apoptosis in human colon carcinoma-

183   derived cells were studied using the TUNEL assay. As shown in Fig. 3, both *Salvia*

184   extracts and RA significantly induced apoptosis in a concentration dependent manner in

185   both HCT15 and CO115 cells. Apoptotic cells in HCT15 increased from 0.4% in the

186   control to 6.6%, 5.8% and 2.5% in SF, SO and RA treatments, respectively, at the

187   higher concentrations tested (Fig. 3A). In CO115 cells, apoptotic cells increased from

188   1.8% in the control to 6.8%, 3.8% and 3.6% in the conditions treated with the higher

189   concentrations tested of SF, SO and RA, respectively (Fig. 3B). Since the basal levels of

190   apoptosis were higher in the CO115 cell line, overall it seems that the HCT15 cells were

191   more sensitive to the extracts and RA. Again, SF extract showed to be more active than

192   SO extract and RA alone.

193   The involvement of caspases 3 and 9 in the apoptosis induction by sage extracts

194   and RA was also studied by western blot. After 24h of treatment with the highest

195   concentrations used of SF, SO and RA, we did not observe cleaved caspase-9 and

196   caspase-3 in either of cell lines, in contrast with the reference compound, staurosporine

197   (data not shown).

198

199   **Effects on MAPK/ERK pathway**

200   The effects of sage extracts and RA for 24h were studied on the MAPK/ERK

201   pathway by western blot. *Salvia* extracts and RA significantly decreased phospho-ERK

202   protein levels in HCT15 cells (Fig. 4A) while no effects were observed in CO115 cells

203   (Fig. 4B). The reference inhibitor of phospho-ERK, PD-98059 (PD) was effective in

204   both cell lines (Fig. 4), in a similar extension than SF, SO and RA in HCT15 cells.

205

206   **Effects on PI3K/Akt pathway**

207   The effects of sage extracts and RA on the expression of phospho-Akt and

208   phospho-PTEN (a negative regulator of PI3K/Akt pathway) were also tested. Phospho-

209   Akt was observed in CO115, however was not detected in HCT15, in medium with and

210   without serum (data not shown). Neither of the *Salvia* extracts nor RA inhibited

211   significantly the expression of phospho-Akt in CO115 cells (Fig. 5A). A significant

212   inhibition of Akt phosphorylation was observed for the reference PI3K inhibitor,

213   wortmannin (W). HCT15 cells expressed phospho-PTEN and this expression was not

214   significantly changed by *Salvia* extracts, RA or W (Fig. 5B). CO115 cells did not

215   express phospho-PTEN or total PTEN, in medium with and without serum (data not

216   shown).

217

218   **Discussion**

219   In order to assess the potential of sage in the control of CRC progression, the

220   antiproliferative and pro-apoptotic effects of *Salvia fruticosa* (SF) and *Salvia officinalis*

221   (SO) water extracts and their main phenolic compound, rosmarinic acid (RA), were

222   studied in two human colon carcinoma-derived cell lines, HCT15 and CO115. Both

223   sage water extracts (SF and SO) were effective in inhibiting proliferation in a

224   concentration-dependent manner in HCT15 but not in CO115 cells. SF, SO and RA

225   induced apoptosis. SF was more effective than SO with regard to both antiproliferative

226   and proapoptotic effects. To identify the bioactive compound behind these effects,

227   sage's major phenolic compound (RA), was tested individually at concentration similar

228   to those present in the extracts. However, RA was found not to have antiproliferative

229   activity but to be proapoptotic in both cell lines, although at less extent than sage

230   extracts. In view of these results, it seems that other active compounds present in the

231   extracts may be responsible for the antiproliferative and proapoptotic effects of SF and

232   SO.

233   The two cell lines used harbor different activating mutations: HCT15 has a

234   KRAS (G13D) activating mutation (28) with potential to constitutively activate both

235   PI3K/Akt and MAPK/ERK pathways, whereas CO115 harbors a BRAF (V599E)

236   mutation (29) which affects the MAPK/ERK pathway. The highest sensitivity of

237   HCT15 could be a result of these genetic differences. HCT15 cells, even though

238   presenting an activating mutation of the RAS oncogene, did not express phospho-Akt

239   possibly as a consequent of the high levels of the strong negative regulator of this

240   pathway, phospho-PTEN, found in this cell line. In these cells, the antiproliferative

241   effects of SF and SO correlate with an inhibition of phospho-ERK. However, RA

242   showed a significant inhibition of phospho-ERK without inhibiting HCT15 cell

243   proliferation. Inhibition of phospho-ERK seems, therefore, not to be the only factor

244   involved in inhibition of cell proliferation in this cell line. Our findings are in agreement

245   with previous studies (6, 31), which have shown that an inhibition of MAPK/ERK

246   pathway in KRAS mutated cell lines is not sufficient to inhibit cell proliferation.

247   Therefore, the KRAS mutated HCT15 cells do not depend exclusively on MAPK/ERK

248   pathway to proliferate and, as a result, SF and SO seem also to be inhibiting other

249   proliferation pathways, which in these cells do not include Akt phosphorylation (Fig. 6).

250   In CO115 cells, where SF and SO did not have antiproliferative effect, there was

251   no inhibition of phospho-ERK or phospho-Akt. RA also did not inhibit proliferation of

252   CO115 cells. However, in contrast to the effects on the other cell line, RA was without

253   effect on phospho-ERK. Inhibition of MAPK/ERK pathway by sage extracts and RA in

254   HCT15 and not CO115 indicates that the effect may be upstream of BRAF and could be

255   on KRAS (Fig. 6). In CO115 cells, a potential inhibition of RAS by sage extracts would

256   not result in antiproliferative effects due to the downstream activating mutation of

257   BRAF (Fig. 6). An inhibition of RAS oncogene has also been recently shown for

258   quercetin, a common natural-occurring phenolic compound (32, 33). It seems that the

259   effects of RA depend on cell type and/or genetic background, since also others have

260   shown that RA decreases ERK phosphorylation in cardiac muscle cells but it is without

261   effect on Akt and ERK in melanoma cells (34, 35).

262   SF, SO and RA induced apoptosis in both cell lines. It seems, however, that

263   under these conditions apoptosis is not dependent on the cleavage of neither caspase-9

264   nor caspase-3 in both cell lines. Nevertheless, some authors have shown that RA

265   promotes apoptosis in human Jurkat cells and HepG2 cells via the mitochondrial

266   pathway and Bcl-2 suppression in which caspases are involved (36-38). Also the

267   mitochondrial pathway was induced by RA in activated T cells from rheumatoid

268   arthritis patients (39). It seems, therefore, that the induction of caspase pathways by RA

269   is cell type specific and/or dependent on concentration and time of exposure which may

270   explain the discrepancy between these and our results. The inhibition of MAPK/ERK

271   pathway may contribute, at least in part, to the effects on apoptosis in HCT15 cells.

272   Besides a possible interaction with KRAS, sage extracts may act as

273   antiproliferative and proapoptotic in these cancer cell lines through their antioxidant

274   activity. It is known that cancer cells produce increased amounts of ROS, in particularly

275 hydrogen peroxide (H_2O_2), which could inhibit protein fosfatases and also be associated

276 with signalling events in MAPK pathways that lead to activation of redox-sensitive

277 transcription factors, mediating cancer cell proliferation and survival (23, 24).

278 Therefore, the radical scavenging activity of the phenolic compounds present in the sage

279 extracts may be reducing the ROS levels in these cancer cells contributing also to a

280 decreased activity of redox-sensitive proliferating pathways, through RAS signalling.

281 Based on RA results, the effects describe in the present study seem, however, not to be

282 totally explained by the antioxidant properties of the sage extracts.

283   In conclusion, our results show that SF and SO water extracts inhibit

284 proliferation and induce apoptosis in colon carcinoma-derived cell lines whereas RA

285 was only effective on the induction of apoptosis. Sage extracts and RA did not affect the

286 PI3K/Akt pathway but inhibited the MAPK/ERK pathway in the KRAS mutated

287 HCT15 cell line. The inhibitory effects of sage extracts on phospho-ERK seem to result

288 from an inhibition of KRAS, upstream to BRAF, since it was not observed in CO115

289 cells. The inhibition of MAPK/ERK by sage extracts seems, however, not to completely

290 explain the inhibition of cell proliferation in HCT15, since RA inhibits phospho-ERK

291 without affecting cell proliferation. These data add *S. fruticosa* and *S. officinalis* to the

292 list of potential sources of new active anticancer compounds useful in particular in

293 tumors with a mutagenic KRAS activation and also suggest their possible use in dietary

294 strategies for the control of CRC progression.

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Appendixes

Figure 1. Chemical structure of rosmarinic acid (RA).

Figure 2. Effect of different concentrations of *Salvia fruticosa* (SF), *Salvia officinalis* (SO) and rosmarinic acid (RA), for 48h, on BrdU incorporation in HCT15 (**A**) and CO115 (**B**) cells. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.
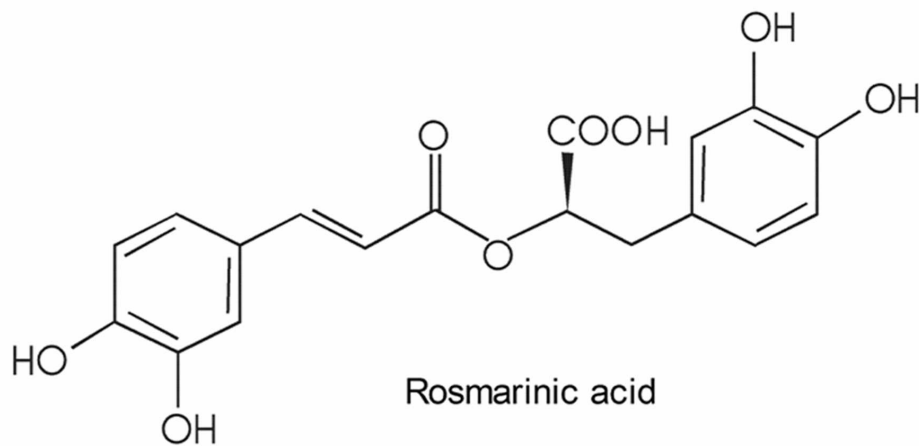
Figure 3. Effect of different concentrations *Salvia fruticosa* (SF), *Salvia officinalis* (SO) and rosmarinic acid (RA) on apoptosis, for 48h, as assessed by the TUNEL assay, of HCT15 (**A**) and CO115 (**B**) cell lines. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001 when compared to control.

Figure 4. Effects of *Salvia fruticosa* 50μg/ml (SF50), *Salvia officinalis* 50μg/ml (SO50) and rosmarinic acid 100μM (RA100) for 24h on the expression of phospho-ERK in HCT15 cells (**A**) and CO115 cells (**B**). PD-98059 50μM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.

Figure 5. Effects of *Salvia fruticosa* 50μg/ml (SF50), *Salvia officinalis* 50μg/ml (SO50) and rosmarinic acid 100μM (RA100) for 24h on the expression of phospho-Akt in CO115 cells (**A**) and phospho-PTEN in HCT15 cells (**B**). Wortmannin 1 μM (W1) was used as a reference inhibitor of PI3K. No phospho-Akt expression was observed in

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HCT15 cells and no PTEN expression was observed in CO115 cells. Values are mean ±

SEM of at least 3 independent experiments. **P≤ 0.01 when compared to control.

Figure 6. Model for the inhibition of ERK phosphorylation by *Salvia fruticosa* (SF),

Salvia officinalis (SO) and rosmarinic acid (RA) in HCT15 but not in CO115 cells. SF,

SO and RA inhibit mutant KRAS leading to a decrease on the levels of phospho-ERK in

HCT15 cell line. In CO115 cells, SF, SO and RA do not change ERK phosphorylation

levels due to a BRAF activating mutation downstream RAS oncogene. The missing

PTEN in CO115 cells and phospho-Akt in HCT15 cells were also observed in this

study.

Chemical structure of rosmarinic acid (RA).
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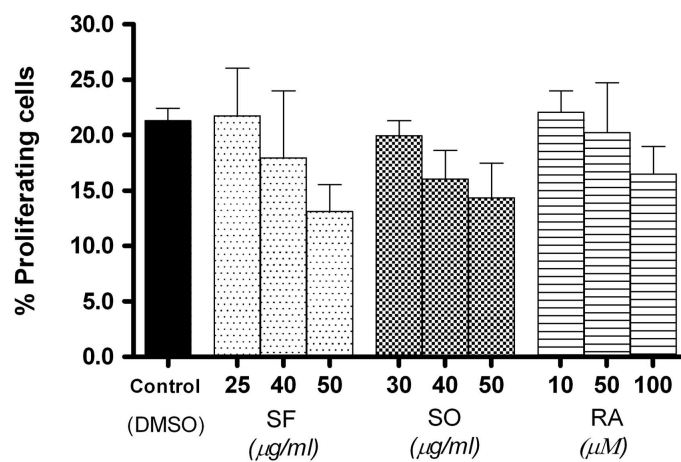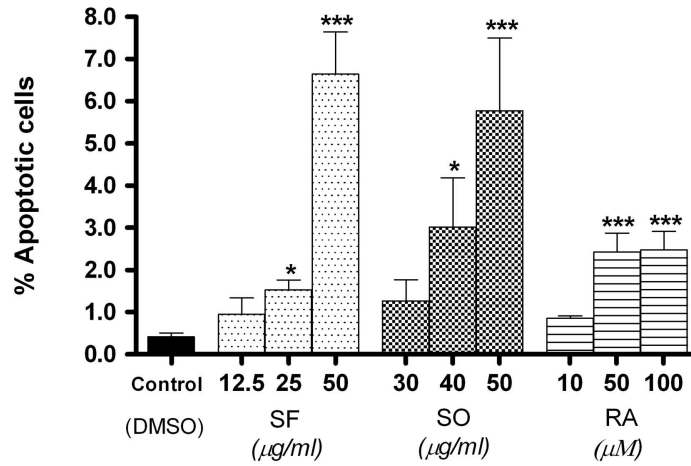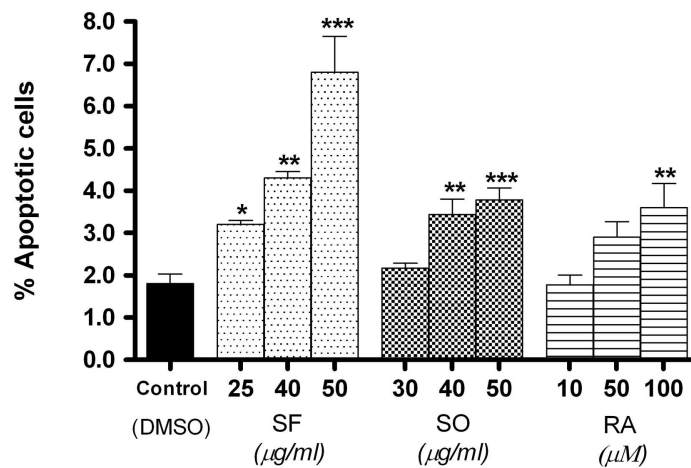A   BrdU incorporation in HCT15
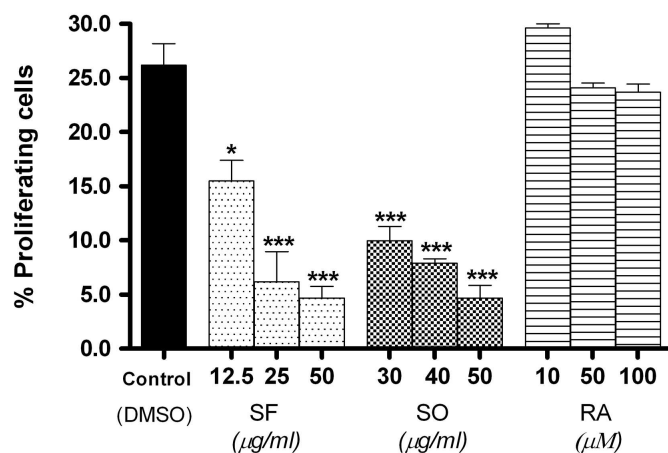
B   BrdU incorporation in CO115

Effect of different concentrations of Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid (RA), for 48h, on BrdU incorporation in HCT15 (A) and CO115 (B) cells. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control.
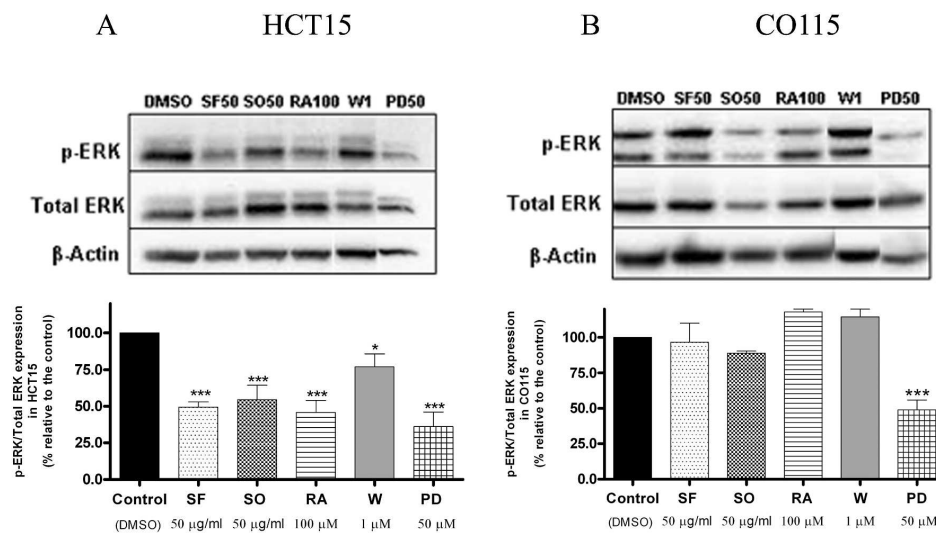80x127mm (600 x 600 DPI)
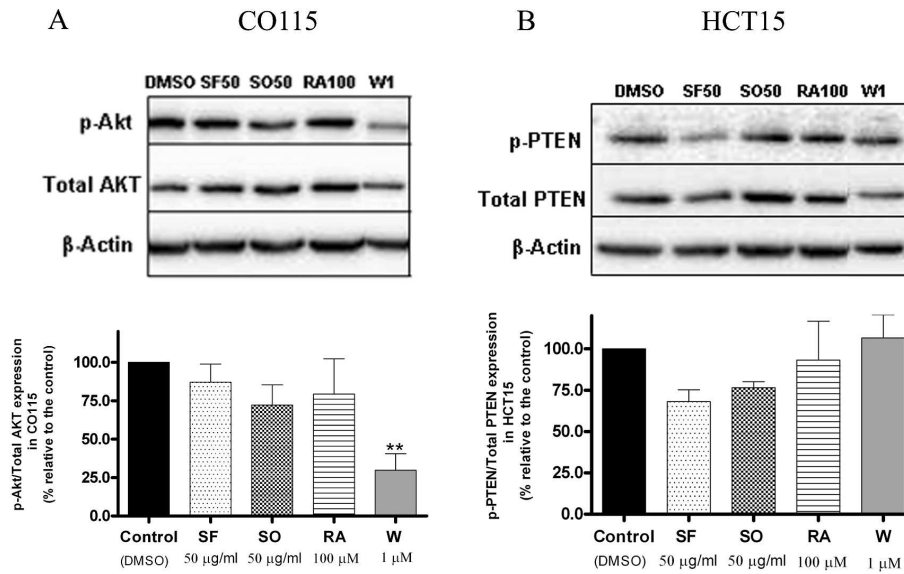
A   TUNEL assay in HCT15

B   TUNEL assay in CO115

Effect of different concentrations Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid
(RA) on apoptosis, for 48h, as assessed by the TUNEL assay, of HCT15 (A) and CO115 (B) cell lines.
Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05, **P≤ 0.01 and ***P≤
0.001 when compared to control.
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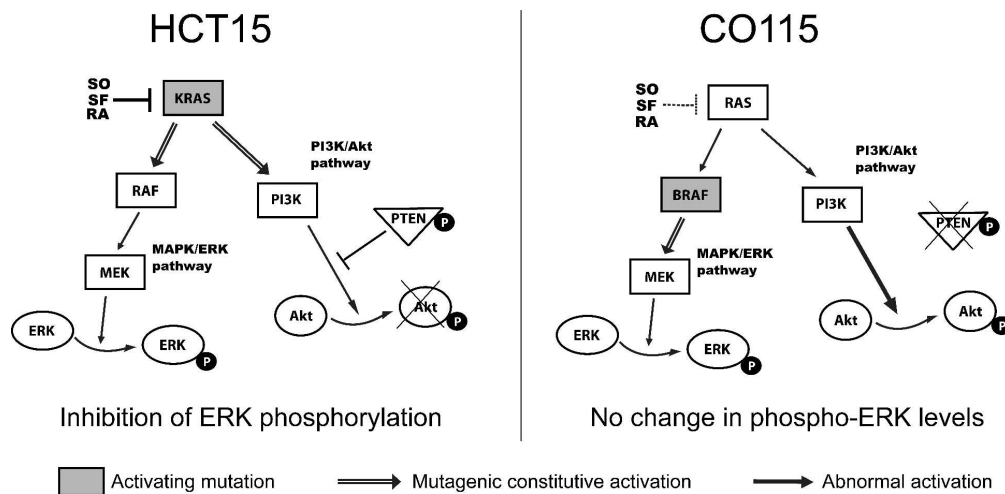

Effects of Salvia fruticosa 50µg/ml (SF50), Salvia officinalis 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-ERK in HCT15 cells (A) and CO115 cells (B). PD-98059 50µM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001 when compared to control. 160x94mm (600 x 600 DPI)

A   CO115   B   HCT15

Effects of Salvia fruticosa 50µg/ml (SF50), Salvia officinalis 50µg/ml (SO50) and rosmarinic acid 100µM (RA100) for 24h on the expression of phospho-Akt in CO115 cells (A) and phospho-PTEN in HCT15 cells (B). Wortmannin 1 µM (W1) was used as a reference inhibitor of PI3K. No phospho-Akt expression was observed in HCT15 cells and no PTEN expression was observed in CO115 cells. Values are mean ± SEM of at least 3 independent experiments. **$P \leq 0.01$ when compared to control.
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Model for the inhibition of ERK phosphorylation by Salvia fruticosa (SF), Salvia officinalis (SO) and rosmarinic acid (RA) in HCT15 but not in CO115 cells. SF, SO and RA inhibit mutant KRAS leading to a decrease on the levels of phospho-ERK in HCT15 cell line. In CO115 cells, SF, SO and RA do not change ERK phosphorylation levels due to a BRAF activating mutation downstream RAS oncogene. The missing PTEN in CO115 cells and phospho-Akt in HCT15 cells were also observed in this study.
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