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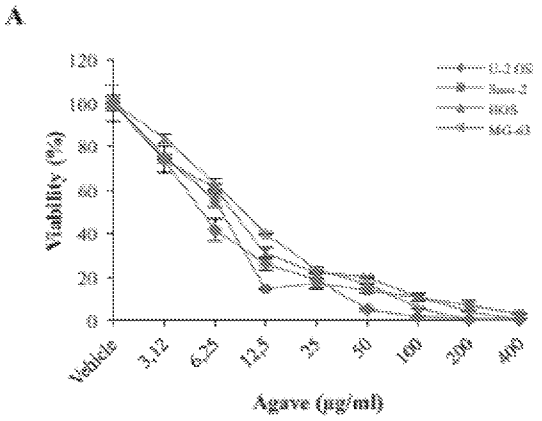

(57) Abstract: The present description relates to new uses of elaborate of Agave and pharmaceutical associations and compositions comprising elaborate of agave, optionally in association with a chemotherapeutic agent and/or an inhibitor of the YAP/TAZ-TEAD pathway in the treatment of other tumours that show deregulation of the YAP and/or TAZ proteins and/or of the mRNA encoding them.

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NEW ACTIVE PRINCIPLES FOR THE TREATMENT OF TUMOURS

DESCRIPTION

The present description relates to new uses of elaborate of agave and pharmaceutical associations and compositions comprising elaborate of agave, optionally in association with a chemotherapeutic agent and/or an inhibitor of the YAP/TAZ-TEAD pathway in the treatment of tumours that show alterations of the regulation of the YAP and/or TAZ proteins.

STATE OF THE PRIOR ART

YAP and TAZ are highly related transcriptional regulators, pervasively activated in human neoplasias. Recent works indicate that YAP/TAZ are essential for cancer initiation or growth in most solid tumours. Their activation induces cancer stem cell attributes, proliferation, chemoresistance, and metastasis. YAP/TAZ are sensors of the structural and mechanical features of the cell microenvironment. A number of cancer-associated extrinsic and intrinsic factors concur to overrule the YAP-inhibiting microenvironment of normal tissues, including changes in mechanotransduction, inflammation, oncogenic signaling, and regulation of the Hippo pathway. Addiction to YAP/TAZ thus potentially represents a central cancer vulnerability that may be exploited therapeutically

Numerous types of tumours in which the responsibility of YAP/TAZ activation in tumour initiation, progression and metastases has been demonstrated are by now reported in the literature. Numerous works in which the YAP/TAZ signature has proven to be present in various tumour types are published in the literature. In particular, the nuclear or cytonuclear localization of said proteins was associated to worse prognosis for the patient. Finally, always in the literature, the correlation between YAP/TAZ and chemoresistance induction is reported. Among tumours in which the YAP/TAZ signature is present, e.g., breast cancer (tumour), lung, colon, liver, pancreatic, thyroid cancers, mesothelioma and osteosarcoma are reported. Osteosarcoma (OS) is the most aggressive type of primary solid tumour developing in bone. While conventional chemotherapy can improve survival rates, the result for patients with metastatic or recurring OS remains poor, therefore new agents and treatment strategies are needed.

Osteosarcoma (OS) is also the most frequent primary tumour affecting bone, typically originating in the ends of long bones of the leg, e.g., femur or tibia, or in arm bones such as the humerus. Less frequently, it

develops in hip bones, shoulders, or in the jawbone, and is always associated with pain increase. Osteosarcoma shows a bimodal incidence curve in connection with age; in fact, it frequently occurs in children and teenagers of age ranging from 10 to 16 years and in adults over 40. Neoplastic cells show a complex karyotype associated with chromosome instability, variation of the number of copies and deregulation of many signaling pathways such as VEGF-R, TGFβ, Wnt/β-catenin, Hippo/YAP, Hedgehog, Notch and PI3K-Akt-mTOR (Abarrategi A, et al. Osteosarcoma: Cells-of-Origin, Cancer Stem Cells, and Targeted Therapies. Stem cells international 2016; 2016: 3631764; Deel MD et al A Review: Molecular Aberrations within Hippo Signaling in Bone and Soft-Tissue Sarcomas. Frontiers in oncology 2015; 5: 190). First-line treatment is combined intravenous chemotherapy (cisplatin (CDDP)/doxorubicin) (See Deel 2015, above). For localized OS patients surgical resection is a fundamental component of therapy; however, if it is not feasible, radiotherapy can be used to improve the prognosis. OS is frequently associated with resistance to chemo- and radiotherapy, due to the presence of cancer stem cell subpopulations and Hippo/YAP signaling alterations (See Deel 2015 above). Therefore, while the overall survival rate for OS has increased, 5-year survival rate for patients with metastatic or recurring disease remained substantially unvaried, at about 20%. New and effective osteosarcoma antitumour strategies, preferably using convenient and less toxic compounds that could pave the way for therapies for osteosarcoma, are urgently needed.

The Hippo pathway is an evolutionary conserved signaling pathway playing a key role in the onset, maintenance of stem cells, regeneration, cancer onset and chemoresistance (Ferraiuolo MS, S.; Blandino, G. The Hippo Pathway. In: Stahl RABaPD (ed). Encyclopedia of Cell Biology, vol. 3, 2016 Elsevier Inc. edn. Academic Press: Waltham, MA, 2016, pp 99-106; Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. Cancer cell 2016; 29: 783-803). The Hippo core comprises protein kinases Lats1/2- (Mob1-Sav1) -Mst1/2 functioning as tumour suppressors. The core kinase cassette induces phosphorylation of Hippo, YAP and TAZ oncogenic transducers, with entailed cytoplasmic retention and/or protein degradation thereof. As YAP and TAZ lack a DNA binding dominion, they interact with various DNA-binding transcription factors, including TEAD/TEF, β-catenin, RUNX1/2 and Smads, to guide the transcription of their oncogenic target genes. Their main function is to regulate cell proliferation, invasion, stem cell maintenance and epythelial-mesenchymal transition (EMT) (Cilloni D, Martinelli G, Messa F, Baccarani M, Saglio G. Nuclear factor kB as a target for new drug development in myeloid malignancies. Haematologica 2007; 92: 1224-1229, Sidana

J, Singh B, Sharma OP. Saponins of Agave: Chemistry and bioactivity. Phytochemistry 2016; 130: 22-46). It is interesting to note that the Hippo pathway has proved to cooperate with p53 signaling to induce apoptosis, DNA damage repair and senescence. Hippo signaling dysregulation and YAP/TAZ hyperexpression or hyperactivation are reported in many types of human cancer, comprising lung, colon, bone and breast cancers.

Previously published works indicate that Agave exhibits some antitumour properties, however the potential molecular mechanisms remain poorly understood. To date, therefore, it is not known how to exploit such properties, nor on which tumours.

The research of new antitumour therapies paved the way for the use of natural compounds, as they are generally less expensive and less toxic compared to conventional chemotherapeutic agents. Therefore, the development of new therapeutic approaches for the treatment of tumours improving existing therapies, possibly enabling to reduce the amounts of chemotherapeutic agents used or increasing their effectiveness, is of fundamental interest.

SUMMARY OF THE INVENTION

The Authors of the present invention have discovered that an elaborate of agave has a marked effect on cell lines of various tumours that show a deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them. The Authors have in fact observed that an elaborate of agave leaves inhibits cell viability, the formation of colonies and cell migration and can induce apoptosis in cell lines of such tumours. Moreover, the Authors of the invention have further discovered that the elaborate of Agave sensitizes the cells of said tumours to treatment with chemotherapeutic agents, such as cisplatin (CDDP), overcoming chemoresistance. The Authors also demonstrated that the elaborate of Agave modulates the Hippo pathway, revealing a marked decrease of YAP and TAZ mRNA and protein expression. The Authors also demonstrated that YAP/TAZ subregulation induced by the elaborate of Agave inhibits OS cell viability and migration. Without being bound by theory, the Authors propose an initial action mechanism wherein the elaborate of Agave induces degradation of the YAP/TAZ protein, followed by a secondary event in which the elaborate inhibits YAP/TAZ transcription and induces degradation thereof in cells.

Therefore, object of the invention are an elaborate of Agave for use in

the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them; the association of an elaborate of Agave with at least one chemotherapeutic agent as such, and in particular for use in the treatment of such tumours, a pharmaceutical composition comprising elaborate of Agave and at least one carrier and/or a pharmaceutically acceptable excipient for use in the treatment of osteosarcoma, a pharmaceutical composition comprising elaborate of agave, at least one chemotherapeutic agent and at least one pharmaceutically acceptable excipient and/or carrier, the above-mentioned composition for use in the treatment of said tumours and process for the preparation of the above-mentioned composition.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1. The elaborate of Agave exerts antitumour effects on osteosarcoma cell lines. (a) Osteosarcoma cell lines were treated with vehicle (EtOH) control or with increasing agave concentrations for 72 hours before being analyzed by ATPlite assay. The percentage of cell viability normalized with respect to control is shown, with values representing the average ± standard deviation (STDEV) of n=3 independent experiments. Wound healing (B-C) and transwell (D, E) assays were carried out on U-2 OS (B, D) and Saos-2 (C, E) cell lines after treatment with elaborate of Agave (3.12 µg/mL) or Vehicle for 24 hours. The histograms represent the percentage of wound width (B, C) or of migrated cells (D, E) normalized with respect to the relevant control. Values represent the mean ± STDEV from n=3 independent experiments, * $p < 0.01$ ** $p < 0.001$. Osteosarcoma U-2 OS (F) and Saos-2 (F) cell lines were treated with Vehicle (CTRL) or elaborate of Agave (3.12 µg/mL) in combination with increasing doses of cisplatin (CDDP) for 72 hours. The percentage of viability of the treatment with elaborate of Agave alone was subtracted from each CDDP or CTRL value (corrected value), then these data were normalized with respect to the corrected value of the CTRL. The values shown represent the mean ± STDEV from n=3 independent experiments. U-2 OS (H) and Saos-2 (I) cell lines were treated with Vehicle, elaborate of Agave (3.12 µg/mL) and/or CDDP (2 µM) for 24 hours after seeding for clonogenic assays. Histograms represent the mean percentage of colonies ± STDEV normalized to vehicle control from n=3 independent experiments, ** $p < 0.001$. The images below the histograms were used for colony counting.

Figure 2. The elaborate of Agave downregulates the oncogenic proteins YAP and TAZ. (A) U-2 OS cells were treated with elaborate of Agave (3.12 µg/mL) for 24, 48 or 72 hours, or EtOH as vehicle control (0 h), then subjected to Western

Blot analysis as indicated. GAPDH was used as loading control. (B) Cell lines Saos-2, HOS and MG-63 were treated with Agave (3.12 µg/mL) or Vehicle for 72 hours, and (C) U-2 OS cells were treated with Vehicle, elaborate of Agave (3.12 µg/mL) and/or CDDP (2 µM) for 72 hours before being subjected to Western Blot analysis as indicated. (D) U-2 OS cells transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ were treated with increasing doses of elaborate of Agave diluted in the Vehicle. The values represent the mean percentage viability ± STDEV for each condition (n=3), determined by ATPlite dosage. (E) U-2 OS and Saos-2 cells were transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ and subjected to clonogenic assays. The histograms represent the mean percentage of colonies ± STDEV normalized with respect to siGFP from n=3 independent experiments, ** p <0,001. The images below the histograms are representative of the stained culture plates used for colony counting. U-2 OS (F) and Saos-2 (G) cells were transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ and subjected to wound healing assay. The histograms show the mean percentage of wound width ± STDEV normalized to siGFP from n=3 independent experiments, * p <0.01 ** p <0.001. The images below the histograms are representative of the wounded areas used to measure wound gap.

Figure 3. The elaborate of Agave induces degradation of the YAP and TAZ proteins. (A) U-2 OS cells were treated with Vehicle (EtOH) or with increasing concentrations of synthetic saponins Diosgenin, Sarsasapogenin and Solasodine for 72 hours before being analyzed by ATPlite assay. The viability percentage of cells normalized for the control is shown, with values representing the mean ± STDEV of n=3 independent experiments. (B) U-2 OS cells were treated with Vehicle, Diosgenin (10 µM), Sarsasapogenin (15 µM) or Solasodine (10 µM) for 72 hours before being subjected to Western Blot analysis as indicated. GAPDH was used as loading control. (C) U-2 OS cells were treated with Vehicle or Agave (3.12 µg/mL) in combination with cycloheximide (CHX, 40 µM) for 16, 24 or 40 hours or DMSO (0 h). Cell extracts were then subjected to Western Blotting with the indicated antibodies and quantitated using Alliance software (UVITEC). The quantitative amount of proteins in each instant for YAP (D) and TAZ (E) was normalized with respect to that of GAPDH. U-2 OS cells were co-transfected with Flag-tagged YAP (F) or TAZ (g) and HA-tagged ubiquitin before being treated with Vehicle or elaborate of Agave (3.12 µg/mL) in combination with

MG-132 (25 μM) for 6 hours. Protein lysates were then subjected to immunoprecipitation (IP) by using anti-IgG or anti-Flag antibodies. Inputs recovered from the IP with anti-IgG were loaded on gel and subjected to immunoblot along with IP samples. Arrows indicate ubiquitinated proteins.

Figure 4. The elaborate of Agave downregulates YAP and TAZ transcription. U-2 OS cells were treated with Vehicle (EtOH), elaborate of Agave (3.12 μg/mL) and/or CDDP (2 μM) for the times indicated before being subjected to analysis with Real Time qPCR. The histograms show the mean level of mRNA ± STDEV of YAP (A), TAZ (B), CTGF (C), ANKRD1 (D) and MCM7 (E) normalized to GAPDH (n=3) * $p < 0.01$ ** $p < 0.001$.

Figure 5. The elaborate of Agave inhibits NF-κB recruitment on YAP and TAZ promoters. (A) Schematic depiction of the relative position of the putative NF-κB binding sites in YAP and TAZ promoters. Different sites are highlighted in different colors, that are consistent with those used for sequences shown in Figure 9 (F-G). U-2 OS cells were treated with Vehicle (EtOH) or elaborate of Agave (3.12 μg/mL) for 72 hours before being analyzed by ChIP. The samples were immunoprecipitated with antibodies against IgG, NF-κB p65, NF-κB p50 or p300 and then subjected to Real time qPCR analysis. The histograms show the mean ± STDEV (n=3) for H1H2BA (B), and the putative NF-κB binding sites inside YAP (C-D) and TAZ (E-F) promoters, ** $p < 0.001$.

Figure 6. The elaborate of Agave downregulates NF-κB transcriptional function. (A) U-2 OS cells were treated with elaborate of Agave (3.12 μg/mL) for 24, 48 or 72 hours, or EtOH as vehicle control (0 h), then subjected to Western Blot analysis as indicated. GAPDH was used as loading control. (B) U-2 OS, Saos-2, HOS and MG-63 cells were treated with elaborate of Agave (3.12 μg/mL) or Vehicle for 72 hours before being subjected to Western Blot analysis. The abundance of NF-κB p65 and NF-κB p50 was quantitated by using Alliance software (UVITEC), and the NF-κB p65/p50 ratio was determined. Relative ratios are shown in (C). Values represent the mean ± STDEV (n=3), with U-2 OS cells treated with vehicle normalized to 1, ** $p < 0.001$. (D) U-2 OS cells were treated with Vehicle or elaborate of Agave (3.12 μg/ml) for 72 hours before being subjected to nuclear/cytoplasmic fractionation followed by Western Blot analysis. (nuclear) H1 and (cytoplasmic) α-Tubulin were used as loading controls. Nuclear/cytoplasmic ratios for NF-κB p105, p50 and p65 were determined (E). Values represent the mean ± STDEV (n=3), with U-2 OS cells treated with vehicle normalized to 1, ** $p < 0.001$. (F, H) U-2 OS cells were treated with Vehicle or elaborate of Agave (3.12 μg/mL) in the absence or presence of IL-6 (100 ng/mL) for 72 hours. The cells were then stained for NF-κB

p105/p50 (F) or p65 (H) with nuclei tagged with DAPI staining. Nuclear and cytoplasmic signals were quantitated using the ImageJ software, and the relative mean ± STDEV for NFKB p105/p50 (G) and NF-kB p65 (I) is shown, with vehicle-treated cells normalized to 1, n=3 * * p <0.001. (J) U-2 OS cells were treated with Vehicle or elaborate of Agave (3.12 µg/mL) for 72 hours before being subjected to Real time qPCR analysis. The mean level of mRNA ± STDEV (n=3) is indicated for each target gene with the vehicle-treated cells normalized to 1, ** p <0.001.

Figure 7. Proposed model of antitumour activity of elaborate of Agave in osteosarcoma. (A) Dimers NF-κB p65:p50 and/or NF-Kb:p65 bind to YAP and TAZ promoters inducing p300 recruitment and transcriptional activation. The activated YAP and TAZ proteins translocate into the nucleus to activate the transcription of oncogenic target genes CTGF, ANKRD1 and MCM7. (B) Treatment with elaborate of Agave promotes ubiquitin-dependent proteasomal degradation of YAP/TAZ, reducing their nuclear translocation. Moreover, the elaborate of Agave downregulates NF-κB p65 and promotes its cytoplasmic sequestration, whereas NF-κB p50 is upregulated and enriched in the nuclear compartment. YAP and TAZ transcription is therefore markedly reduced, as well as their targets downstream.

Figure 8. H1299 (A), A549 (B), MSTO-211H (C), MPP-89 (D), MDA-MB-231 (E) and SUM-159PT (F) cells were treated with vehicle (EtOH) or increasing concentrations of elaborate of Agave for 72 hours before being analyzed with the ATPlite assay. The viability percentage of cells normalized for the control is shown, with values representing the mean ± STDEV of n=3 independent experiments.

Figure 9. Western Blot analysis of U-2 OS (A) and Saos-2 (B) cells transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ. U-2 OS cells were treated with Vehicle (EtOH), Agave (3.12 µg/mL) and/or CDDP (2 µM) for 72 hours before being subjected to Real time qPCR analysis. The histograms show the mean level of mRNA ± STDEV (n=3) for TEAD1 (C), LATS1 (D) and LATS2 (E), with the vehicle-treated cells normalized to 1, * p <0.01. Putative NF-κB binding sites were analyzed inside the YAP and TAZ promoters by using LASAGNA-Search 2.0 software. Some sequences are present on both DNA filaments and on more overlapped sites. Sites nearer to the TATA/TBP box, which might be transcriptionally more active, were preferentially selected.

GLOSSARY

Abbreviations:

Cisplatin (CDDP); Cycloheximide (CHX); Epythelial-Mesenchymal Transition (EMT); Nuclear factor kappa-light chain enhancer of activated B cells (NF-κB); Osteosarcoma (OS); half maximal effective concentration (EC50); half maximal lethal concentration (LC50); cocktail of protein inhibitors (CI); 4 ', 6-diamidino-2-phenylindole (DAPI).

-By “agave” for the purposes of the present description it is meant a plant selected from:

Agave sisalana
Agave americana L.
Agave americana var. *expansa* (Jacobi) Gentry
Agave asperrima Jacobi
Agave asperrima subsp. *maderensis* (Gentry) B.Ullrich
Agave asperrima subsp. *potosiensis* (Gentry) B.Ullrich
Agave asperrima subsp. *zarcensis* (Gentry) B.Ullrich
Agave atrovirens Karw. ex Salm-Dyck
Agave attenuata Salm-Dyck
Agave attenuata subsp. *dentata* (J.Verschaff.) B.Ullrich
Agave brevispina Trel.
Agave cocui Trel.
Agave durangensis Gentry
Agave intermixta Trel.
Agave karatto Mill.
Agave lechuguilla Torr.
Agave mapisaga Trel.
Agave salmiana Otto ex Salm-Dyck
Agave salmiana var. *angustifolia* A.Berger
Agave salmiana subsp. *crassispina* (Trel.) Gentry
Agave salmiana var. *ferox* (K.Koch) Gentry
Agave shrevei Gentry
Agave striata Zucc.
Agave tequilana F.A.C.Weber
Agave victoriae-reginae T.Moore
Agave vivipara L.
Agave wocomahi Gentry

YAP: Gene ID: 10413

TAZ: Gene ID: 6901

- By the wording "tumours that show deregulation of the YAP/TAZ proteins and/or of the mRNA that encodes them" it is meant the definition commonly used in the literature for said tumours, i.e., tumours in which the expression of YAP and/or TAZ proteins and/or of the mRNA that encodes them is deregulated (at a transcriptional, translational and/or post-translational level) with respect to healthy cells surrounding such tumours. The wording "tumours that show (or exhibit) deregulation of the YAP/TAZ proteins and/or of the mRNA that encodes them" can be replaced anywhere in the present description and in the claims by the wording "tumours characterised by a deregulation of the YAP/TAZ proteins and/or of the mRNA that encodes them", by "tumours characterised by the genetic signature YAP/TAZ". The subphrase "deregulation of the YAP/TAZ proteins and/or of the mRNA that encodes them" can be replaced anywhere in the present description and in the claims by "deregulation of YAP and/or TAZ proteins expression at a transcriptional, translational and/or post-translational level".

Numerous ways to detect said deregulation are reported in the literature, e.g., in PNAS | September 11, 2012 | vol. 109 | no. 37 "The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain" John M. Lamar et al.; Cancer Cell 29, June 13, 2016 "YAP/TAZ at the Roots of Cancer" F. Zanconato et al; The Journal of Clinical Investigation Volume 127 Number 9 September 2017 "YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation" Jongshin Kim et al.

By "genetic signature of a tumour" it is meant a set of genes showing a gene expression pattern altered in tumour cells with respect to the healthy cells in the same type of tissue.

As to tumours that show alteration of the YAP/TAZ regulation, the genetic signature can be determined at least by the following genes:

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B ; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795.

The wording "inhibitor of the YAP/TAZ-TEAD pathway" indicates a

molecule, a substance, a group of substances, an elaborate, a compound inhibiting at any stage thereof the YAP/TAZ-TEAD pathway, and therefore the activity of one of these proteins or intra- and intercellular responses triggered thereby. Among the YAP/TAZ-TEAD inhibitors numerous small molecules are known, e.g., small molecules containing an oxime pharmacophore, antagonists of the receptors of the above-mentioned proteins, antitumour agents, immunotherapeutic agents and other molecules known in the literature to have such activity.

For the purposes of the present invention, by "elaborate of agave" it is meant a product obtained from the processing of agave plant, which may be a product obtained by milling of the whole plant or of parts thereof, like, e.g., the leaves, in which said parts are preferably dehydrated or dried; a product obtained by milling, grinding, pestle treatment, of the whole plant or of parts thereof, e.g., formulated in the form of pulp, which could be further processed (e.g., dried), or an extract obtained from a plant, preferably leaves, of agave.

Anywhere in the present invention the term "elaborate" may be replaced by "product (obtained/obtainable) from the processing of an agave plant" or "product (obtained/obtainable) from the processing of agave leaves", or directly from "agave".

DETAILED DESCRIPTION OF THE INVENTION

The Authors of the invention have surprisingly discovered that the elaborate of Agave as defined hereinafter has an antitumour effect and an effect of reduction of chemoresistance on tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

One of the major problems in the treatment of tumours is precisely chemoresistance. The Authors of the invention have discovered that the elaborate of agave, besides having *per se* an antitumour effect, acts on the regulation of YAP/TAZ proteins, inducing a decrease to chemoresistance in cells treated with said elaborate. The experimental data obtained by the Authors of the invention demonstrate an effectiveness of the elaborate on various components of the Hippo pathway, resulting, in actual fact, in an inhibition of chemoresistance in treated cells.

This discovery is of fundamental importance and opens new perspectives to the therapeutic treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them with the sole elaborate of Agave or, more advantageously, with a simultaneous or sequential association of elaborate of Agave and chemotherapeutic agent.

As demonstrated by the Authors of the invention, in fact, the elaborate of Agave reduces cell viability of the tumour cells that show deregulation of YAP/TAZ expression, weakens migration and the formation of colonies and sensitizes to

treatment with a chemotherapeutic agent, in various tumour lines assayed. As shown in the figures and reported in the Experimental Examples section, the action mechanism triggered by the treatment with elaborate of Agave downregulates YAP and TAZ in the Hippo pathway.

Of particular importance the discovery, by the Authors of the invention, that the elaborate of agave, besides having the above-described antitumour effects, induces an apoptotic response (that seems to be p53-indipendent) and enhances the cytotoxicity of the chemotherapeutic agents assayed. What discovered by the Authors supports the YAP and TAZ oncogenic role in assayed cell lines. These proteins are often overexpressed in samples of different types of human tumours, also correlating with patients' survival expectancies. In fact, it is reported in the literature that a nuclear or nucleocytoplasmic localization of these proteins is correlated with a more fatal prognosis for the patients, as compared to patients that do not exhibit said localization.

The elaborate of Agave is a phytocomplex comprising numerous substances, among which also saponins which are known to have antimicrobial, pro-apoptotic, immunomodulatory, neuroprotective, antiproliferative and antimigratory effects on numerous tumour cell lines. Among saponins activity, the induction of p53-dependent apoptosis is reported. The experiments carried out by the Authors of the invention demonstrate that the cytotoxic effect of the elaborate of agave, thanks to its combination of active components that can activate/inhibit multiple signal pathways simultaneously, is in any case superior to that exerted by saponins.

In fact, the studies carried out by the Authors of the invention show that the elaborate of Agave activates numerous mechanisms contributing, as a whole, to its antitumour activity and to its ability to enhance the cytotoxic effect of the chemotherapeutic agent, by sensitizing cells to the chemotherapeutic agent.

The data produced in the present description indicate that YAP/TAZ are mediators of the antiproliferative, antimigratory and proapoptotic effects induced by the elaborate of Agave in the cell lines analyzed, like, e.g., osteosarcoma (OS) cell lines, and possibly in OS of animals and human patients. In particular, the results show that the elaborate of Agave induces YAP/TAZ protein degradation as an early event, and subsequently alters YAP and TAZ transcription by NF-κB inactivation (Figure 7). YAP/TAZ downregulation triggered by the elaborate of Agave translates into a

proapoptotic, antimigratory phenotype, with a greater chemosensitivity to the chemotherapeutic agent. The tumours that show YAP/TAZ deregulation as defined herein can therefore be attacked by treatment with elaborate of Agave alone, or optionally in combination with drugs inhibiting the YAP/TAZ-TEAD complexes, guiding the transcription of proliferative and anti-apoptotic genes. The treatment with elaborate of Agave can also be combined in association with the treatment with chemotherapeutic agent, and optionally in combination also with the treatment with said YAP/TAZ-TEAD inhibiting drugs. An example of known YAP/TAZ-TEAD inhibitors is given by verteporfin, a photosensitizer that is used clinically to treat macular degeneration, which proved to inhibit YAP-induced excessive liver growth in animal models; the peptides which mimic VGLL4; dasatinib and pazopanib, which block YAP/TAZ translocation into nucleus; dobutamine, which facilitates YAP exit from nucleus. In the pharmaceutical field, new YAP/TAZ-TEAD-inhibiting therapeutic compounds are expected to be released on the market. Natural compounds afford advantages compared to traditional chemotherapies, as said compounds typically exhibit low toxicity, are associated with low production costs, and afford the potential for multifaceted action mechanisms.

Therefore, object of the invention is an elaborate of Agave for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

By “elaborate of Agave” for the purposes of the invention it is meant a pharmaceutical form enabling direct administration thereof or of compositions comprising it, both locally and systemically. Should the technician in the field wish to set up solid forms for oral administration, he/she could resort to powder milling to different grain sizes, or to a direct micronization thereof. The powder could in turn be granulated with the possible addition of suitable pharmaceutically acceptable excipients in order to obtain granules, capsules or tablets, plain or coated. For the preparation of liquid forms, the technician in the field can resort to an extract of agave leaves, if necessary using pharmaceutically acceptable solvents, such as, e.g., water and ethanol. The extract can, for instance, be dried by evaporation, lyophilisation or spray-dryer. Possible embodiments for administration in liquid form include, without being limited thereto, syrups, drops or solutions for oral use. In one embodiment, the elaborate will be formulated in preparations for topical use such as gels, emulsions, ointments and solutions, by use of pharmaceutically accepted excipients. Using elaborates such as extracts or powders as described above or derived from extracts, by the known techniques, injectable forms may be set up comprising both liquid forms and solid forms to be diluted before administration with

water or physiological solution, e.g., for injectable preparations. In one embodiment, for the preparation of the above-described compositions there may be used, for instance, a ground agave leaf flour, a very fine agave leaf powder, like, e.g. an impalpable powder, a micronized agave leaf powder, a granule, an agave leaf extract, like e.g. an agave leaf hydroalcoholic extract, preferably lyophilised, a leaf gel extract, preferably lyophilised, and the like.

Therefore, object of the invention are pharmaceutical compositions comprising the elaborate, as sole active principle or in association with other active principles having antitumour activity, as hereto described for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

In one embodiment of the invention, the elaborate of Agave may be obtained by the following steps:

- Agave leaves are dried, e.g., by keeping them in a heater at about 40°C for 10-20 days (e.g., 15);

- the dried sample is then ground with a mill, to obtain a very fine powder.

The powder so obtained can also be micronized, thereby providing an elaborate useful for pharmaceutical preparations. In one embodiment, the elaborate of Agave in any one of the above-defined embodiments is used in association with at least one chemotherapeutic agent, chemotherapeutic agents suitable in order to carry out the invention. A non-binding example of said chemotherapeutic agents is represented by cisplatin, iphosphamide, doxorubicin, methotrexate.

The association with chemotherapeutic agent could be further strengthened by a concurrent or sequential association, with an immunotherapeutic agent according to standard antitumour therapies.

The selection of the chemotherapeutic agent and of the possible immunotherapeutic agent could vary depending on the tumour treated according to the protocols commonly used on each tumour type.

Therefore, according to one embodiment of the invention, current conventional antitumour therapies could be strengthened and made more effective by an association of said therapies with a concurrent or sequential administration of elaborate of Agave according to the invention.

Given the effects disclosed in the present description, of the administration of the elaborate of agave to cell lines of tumours that show YAP/TAZ deregulation, and given the sensitization to chemotherapy induced

by said administration, it is evident how an association between current conventional therapies and the administration of elaborate of Agave is extremely advantageous.

Moreover, according to a further embodiment, given the effect exerted by the elaborate of Agave precisely on YAP/TAZ expression and regulation reported herein, said elaborate may advantageously be used in association with at least one inhibitor of the YAP/TAZ-TEAD pathway, optionally also in association with a chemo/immuno-therapeutic regimen as reported above.

In order to carry out the invention, said inhibitor may be selected from verteporfin, peptides which mimic VGLL4, dasatinib, pazopanib, dobutamine.

In a further embodiment, the elaborate may be in association with any one of the above-defined chemotherapeutic agents and any one of the YAP/TAZ-TEAD pathway inhibitors in any possible combination.

According to the present invention, by "tumours that show deregulation of the YAP and/or TAZ proteins and/or of the mRNA that encodes them" tumours are meant in whose cells the expression of YAP/TAZ proteins is deregulated at a transcriptional or translational or post-translational level, or a combination thereof.

E.g., said deregulation can appear as nuclear or nucleoplasmic localization of at least one of said proteins.

Said tumours are easily identifiable by direct analysis of YAP/TAZ expression in tumour tissues and in healthy ones surrounding them (e.g., by immunohistochemistry), or by analysis and comparative quantitation of the mRNA encoding such proteins in said tumours and in healthy tissues surrounding them, or by reading the genetic signature characteristic of such tumours, or according to any one of the modes reported in the literature or by gene expression analysis

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795

wherein, an abnormal expression of said genes in the tumour mass with respect to the healthy surrounding tissue is considered to be a genetic signature for the abovementioned tumours.

By way of example, the signature may be identified by RNA extraction, cDNA synthesis by reverse transcription and amplification and quantitation thereof by real-time PCR with the suitable primers for the above-indicated genes, using one or more housekeeping genes like, e.g., GAPDH or beta-actin as control.

According to the present invention, a non-limiting example of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them is represented by myeloid tumours, breast cancer (tumour), triple negatives included, lung cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer.

According to the invention, an elaborate of Agave useful as claimed herein and as component of the associations or compositions claimed herein may be obtained by any process commonly used in the state of the art, even by simple crushing and powdering of dried agave leaves.

Therefore, object of the invention is also a composition comprising as sole active ingredient an elaborate of Agave in any one of the embodiments indicated in the present description for use in the treatment that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them as defined above.

Object of the invention is also an association di elaborate of Agave in any one of the embodiments provided in the present description, at least one chemotherapeutic and/or immunotherapeutic agent as indicated in any one of the embodiments provided in the present description and/or at least one inhibitor of the YAP/TAZ-TEAD pathway as defined above and as indicated in any one of the embodiments provided in the present description.

Therefore, the association according to the invention could be represented by a concomitant or sequential association in its administration, of elaborate of Agave and chemotherapeutic agent, of elaborate of Agave and immunotherapeutic agent, of elaborate of Agave and chemotherapeutic and immunotherapeutic agent, of elaborate of Agave and inhibitor of the YAP/TAZ-TEAD pathway, of elaborate of Agave and inhibitor of the YAP/TAZ-TEAD pathway and chemotherapeutic agent or of elaborate of agave, inhibitor of the YAP/TAZ-TEAD pathway, chemotherapeutic agent and immunotherapeutic agent.

In a particularly advantageous embodiment, the elaborate of Agave could be associated with treatment regimens of the abovementioned tumours

commonly used by virtue of its ability to restore sensitivity to chemotherapeutic agents.

According to one embodiment, the association can therefore comprise an elaborate of Agave in any embodiment provided herein and a chemotherapeutic agent selected from those indicated herein, like, e.g., cisplatin, iphosphamide, doxorubicin, methotrexate.

According to a further embodiment, the association can comprise an elaborate of Agave in any embodiment provided herein and an inhibitor of the YAP/TAZ-TEAD pathway as defined herein, like e.g. verteporfin, peptides which mimic VGLL4, dasatinib, pazopanib, dobutamine.

Furthermore, the association according to the invention can be triple and therefore comprise the elaborate in any one of the embodiments defined herein, a chemotherapeutic agent selected among those indicated above and an inhibitor of the YAP/TAZ-TEAD pathway selected from those indicated above.

Object of the invention is also the association according to any one of the embodiments provided above for medical use.

In particular, there can be used the association of the invention that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them as abovedefined.

As mentioned above, a non-limiting example of such tumours is represented by myeloid tumours, breast cancer (tumour), lung cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer, wherein breast cancer advantageously includes triple negative.

Furthermore, object of the invention is a pharmaceutical composition comprising an association as described according to any one of the embodiments provided in the present description and at least one pharmaceutically acceptable excipient and/or carrier.

The expert in the field will be able to select carriers and excipients according to the form of pharmaceutical composition desired.

According to the invention, the association between the elaborate of Agave and the chemotherapeutic agent can also be carried out by oral administration of the elaborate of agave and intravenous administration of the chemotherapeutic agent and/or the immunotherapeutic agent.

Object of the invention is also the pharmaceutical composition as defined herein in any of its embodiments for medical use, in particular for use in the

treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them as hereto defined.

Therefore, in one of the embodiments of the invention, such tumours may be selected from myeloid tumours, breast cancer, lung cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer, wherein breast cancer includes triple negative. Object of the invention is also a process for the preparation of a pharmaceutical composition as defined in the present description, wherein an elaborate of Agave prepared according to any one of the above-described methods is put into a formulation with at least one chemotherapeutic agent and/or immunotherapeutic agent as above-defined and/or at least one inhibitor of the YAP/TAZ-TEAD pathway as above-defined, and at least one pharmaceutically acceptable carrier and/or excipient. Said composition can also be formulated in the form of kit of parts wherein one or more of the above-described pharmacologically active agents (elaborate, chemotherapeutic agent, immunotherapeutic agent, inhibitor) are provided separately and administered to the patient simultaneously or sequentially, also via administration pathways different among them (e.g., agave via OS chemotherapeutic/immunotherapeutic agent, or other active principle intravenously) as however normally done in combined therapies (e.g., chemotherapeutic/immunotherapeutic agent).

Finally, object of the invention is a therapeutic method for the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them as defined hereto, wherein a therapeutically effective dosage of elaborate of Agave according to any of the embodiments provided in the present description is administered, optionally in association with a therapeutically effective dosage of a chemotherapeutic/immunotherapeutic agent as defined above and/or of an inhibitor of the YAP/TAZ-TEAD pathway as defined above to a patient in need thereof.

The physician will define the effective dosage on the basis of patient's weight, age, gender and health state.

EXAMPLES

All cell lines used are commercial cell lines obtained from ATCC (Manassas, USA).

OS: Osteosarcoma
U-2 OS, ATCC® HTB-96™
Saos-2, ATCC® CRL-7939™
HOS, ATCC® CRL-1543™
MG-63, ATCC® CRL-1427™

Lung cancer
H1299, ATCC® CRL-5803™
A549 ATCC® CCL-185™

Breast cancer
MDA-MB-231 ATCC® HTB-26™
SUM-159-PT, ATCC® TCP-1003™

Mesothelioma
MSTO-211H ATCC® CRL-2081™
MPP-89 ATCC® CRL-5830™

1. Preparation of the elaborate of agave

Agave sisalana leaves were kept in heater at 40°C for 15 days

-the dried sample was milled with a Retsch brand laboratory cutting mill, SM100 model.

Then elaborate so obtained, in the form of very fine powder, was used in the experiments reported hereinafter.

2. Materials and methods

2.1. Cell culture and reagents

Cell lines were obtained from ATCC (Manassas, USA) and cultured in DMEM (U-2 OS, Saos-2, HOS, MG-63, H1299, A549 and MDA-MB-231) or DMEM-F12 (SUM-159-PT, MSTO-211H and MPP-89) (Gibco, Life Technologies, Carlsbad, CA, USA) completed with 10% FBS (Life Technologies), 100 units/ml of penicillin and 100 µg/mL of streptomycin (Life Technologies). Cell lines were cultivated at 37°C in 5% CO_2. DPBS (EuroClone, Milan, Italy) and 0.05% trypsin (GE Healthcare Hyclone, Little Chalfont, United Kingdom) were used to wash and detach cells. The total elaborate of agave was used in lyophilised form and resuspended to generate a mother solution at 50 mg/ml in absolute EtOH. The elaborate of Agave was obtained from frozen leaf samples of *Agave sisalana*. The saponins diosgenin (Cat. D1634), Sarsasapogenin (Cat. S8534) and Solasodine (Cat. SML1141) were

purchased from Sigma (Saint Louis, Missouri, U.S.A.) and resuspended in absolute EtOH at 10 mM. Cisplatin (CDDP) dissolved in saline was provided by the Pharmacy of the "*Istituto Nazionale Tumori Regina Elena*" of Rome. DAPI (4', 6-diamidino-2- phenylindole, dihydrochloride, Sigma Cat. 32670) was used to stain cell nuclei. The cocktails of protease inhibitors (CI) (Cat. P8340), MG-132 (Z-Leu-Leu-Leu-al, Cat. C2211), Cicloheximide (CHX) (Cat. 01810) and IL-6 (Cat. I1395) were purchased from Sigma. CHX was dissolved in DMSO solution (Cat. 907201418, Carlo Erba, Cornaredo, Milan, Italy).

2.2. Cell transfection

Transfections were performed by using Lipofectamine 2000 (for plasmids) and Lipofectamine RNAiMax (for siRNA) (Life Technologies) following the manufacturer's instructions.

siRNAs, siGFP, siYAP and siTAZ were purchased from Eurofins MWG (Ebersberg, Germany). In order to exclude nonspecific effects, YAP and TAZ silencing was performed beforehand with two different siRNAs. Representative data are shown in figures. Plasmids used were: pCDNA3-YAP-Flag 66, pCS2-TAZ-Flag 67 and pCS2-Ub-HA 68.

2.3. Viability assay

Cells (800 per well) were seeded in 96-well plates in 200 µl of medium. After 24 hours, cells were treated for the indicated times. Where indicated, gene silencing was performed by transfecting cells in suspension immediately before plating for experiments. Cell viability assays were performed by using the ATPlite assay (Perkin Elmer, Massachusetts, USA) on the basis of the manufacturer's instructions. Plates were assessed by using an EnSpire Technology (Perkin Elmer) microplate reader.

2.4. Clonogenic assay

Cells (1000) were plated in 6-well plates (Corning-Costar, Tewksbury, MA, USA) and treated as indicated. New media were added (25% of overall volume) with treatments every 3 days. After 15-21 days, cells were stained with crystal violet and colonies were counted by using OpenCFU Free Open-299 Source Software.

2.5. Wound-healing migration assay

The wound-healing assay was performed as described in Materials and Methods of the publication by Pulito C et al. "Metformin-induced ablation of microRNA 21-5p releases Sestrin-1 and CAB39L antitumoral activities" Cell discovery 2017; 3: 17022. Migration progression was photographed at

times t = 0 and t = 24 hours by using an optical microscope. Wound gap was measured and data were normalized to t = 0 points.

2.6. Transwell migration assay

Migration assays were performed as described in Materials and Methods of the publication Mori F et al. "Multitargeting activity of miR-24 inhibits long-term melatonin anticancer effects" Oncotarget 2016; 7: 20532-20548. Treatments with elaborate of Agave or vehicle were performed for 24 hours. Cells in migration were counted with a fluorescence confocal laser scanning microscope Zeiss LSM 510 (Zeiss, Wetzlar, Germany).

2.7. Cell extracts, immunoprecipitation and Western blotting

Cell lysis, protein quantitation, immunoprecipitation (IP) and Western Blot analysis were performed as described in Materials and Methods of the publication by Pulito C et al. "Metformin-induced ablation of microRNA 21-5p releases Sestrin-1 and CAB39L antitumoral activities" Cell discovery 2017; 3: 17022. Primary antibodies are listed in Table 1 below:

Table 1

Antibody	Code	Application
anti-GAPDH	sc-47724, Santa Cruz Biotechnology	WB
anti-Lats1	Cat. 9153, Cell Signaling Technology	WB
anti-YAP1	ab56701, Abcam	WB
anti-TAZ (anti-WWTR1)	Cat. HPA007415, Sigma	WB
anti-TEAD1 (anti-TEF-1)	Cat. 610923, BD-Transduction Laboratories	WB
anti-NF-κB p65	Cat. D14E12, Cell Signaling Technology	WB, IF
anti-NF-κB p65	C-20 sc-372, Santa Cruz Biotechnology	ChIP
anti-NF-κB p105/p50	H-119 sc-7178, Santa Cruz Biotechnology	WB, IF, ChIP
anti-HA-probe	sc-7392, Santa Cruz Biotechnology	WB
anti-Bax	N-20 sc-493, Santa Cruz Biotechnology	WB
anti-caspase 3	Cat. 31A1067, Enzo Life Science	WB
anti-PARP	Cat. 9542, Cell Signaling Technology	WB
anti-FLAG	F1804 clone M2, Sigma	IP
anti-αTubulin	B-7 sc-5286, Santa Cruz Biotechnology	WB
anti-Histone H1	N-16 sc-34464, Santa Cruz Biotechnology	WB
anti-p300	N-15 sc-584, Santa Cruz Biotechnology	ChIP

For chemoluminescence detection, ECL reagent (Amersham, GE Healthcare, Piscataway, NJ, USA) was used.

2.8. RNA extraction, reverse transcription and quantitative Real-Time PCR

RNA was extracted, retro-transcribed and subjected to quantitative real-time PCR (qPCR) as described in Materials and Methods of the publication by Mori F et

al. "Multitargeting activity of miR-24 inhibits long-term melatonin anticancer effects" Oncotarget 2016; 7: 20532-20548. For detection, QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems) was used. Data were analyzed by using the relative standard curve method and normalized to GAPDH.

2.9. Protein stability assay

Cells were treated with Vehicle or elaborate of Agave as indicated, and concomitantly treated with CHX 40 μM for the indicated times. Cells were subsequently lysed and subjected to Western Blotting as previously described.

a) abnormal nuclear localization of YAP and TAZ

b) high expression of the genetic signature of YAP and TAZ

2.10. Ubiquitination assay

$1.6x10^6$ cells were transfected with 5 μg of the plasmids specified as described above and simultaneously treated with Vehicle or elaborate of Agave as indicated. After 18 hours, cells were treated with 25 μM of MG-132 for additional 6 hours. Protein extracts were immunoprecipitated as described and subjected to Western Blotting. Antibody specifications are listed in the Table above.

2.11. Extraction of nuclear/cytoplasmic proteins

Harvested and washed cells were lysed in buffer A (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA pH 8.1 mM DDT, 0.5% NP -40, 1 mM CI) and centrifuged at 9800 RCF, for 30 minutes at 4°C. Cytoplasmic extracts (supernatant) were transferred into a new test tube and nuclear pellets washed twice in buffer A before being lysed in buffer B (20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA pH 8, 1 mM DDT, 1 mM CI). The lysates were then centrifuged at 19000 RCF for 10 minutes at 4°C and the supernatants recovered. Nuclear and cytoplasmic extracts were processed as described above for Western Blot analysis.

2.12. Immunofluorescent staining

Samples were treated as described in Materials and Methods (Pulito C et al. Cynara scolymus affects malignant pleural mesothelioma by promoting apoptosis and restraining invasion. Oncotarget 2015; 6: 18134-18150); and treated as indicated for 72 hours. IL-6 treatments were performed by using 100 ng/ml for 30 minutes before recovering the cells. Antibody specifications are listed in the above Table.

2.13. Chromatin immunoprecipitation assay (ChIP)

Cells were crosslinked, sonicated, and the chromatin in solution was immunoprecipitated with the indicated antibody, or with IgG (Santa Cruz Biotechnology) as negative control, as described in Materials and Methods of Di Agostino S et al. "YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins" EMBO reports 2016; 17: 188-201. Immunoprecipitated and purified chromatin was subjected to Real time qPCR. Data were normalized to the amount of chromatin input, and these data were further normalized by subtracting the signal of the Real time qPCR of a negative control sequence that was not modulated (H1H2BA).

Promoter analysis. The FASTA sequences of human YAP1 promoters (NM_001130145) and WWTR1 (TAZ) (NM_015472) (4000 bp upstream of the TSS) were downloaded from the online UCSC Genome Browser database and pasted in LASAGNA-Search 2.0 to identify the binding sites of the transcription factor envisaged. TRANSFAC matrices were used for analysis.

2.14. Statistical analysis

All experiments were independently performed at least thrice, and the more representative results are shown. No sample was excluded from the analysis. All p values were determined by using two-tailed t-test and statistical significance was set to * $p < 0.01$, ** $p < 0.001$.

3. Experimental data

3.1 The elaborate of Agave reduces cell viability, colony formation, cell migration, and sensitizes cells to CDDP

Performing a cell viability assay by testing the elaborate of Agave as described herein on U-2 OS, Saos-2 OS, HOS and MG-63 cell lines, The Authors have noticed that said elaborate reduced the viability of tumour cells assayed. The natural elaborate of Agave showed the greatest reduction of cell viability. Dose-response analyses were performed by using the treatment with elaborate of Agave for 72 hours (Figure 1A). Table 2 below shows the concentration of said elaborate required to produce 50% of the maximal effect (EC50) and the lethal concentration 50 (LC50) for each cell line. EC50 values ranged from 3.67 µg/mL in OS U-2 cells to 6.16 µg/mL in HOS cells.

	U-2 OS		Saos-2		HOS		MG-63	
Phytocompound	EC50 (µg/mL)	LC50 (µg/mL)	EC50 (µg/mL)	LC50 (µg/mL)	EC50 (µg/mL)	LC50 (µg/mL)	EC50 (µg/mL)	LC50 (µg/mL)

Elaborate of agave	3.67	5.21	4.8	6.3	6.16	9.38	4.39	7.81

Table 2: the treatment with Agave reduces cell viability. EC50 and LC50 for osteosarcoma lines treated with elaborate of agave, calculated by using Compusyn software from the dose-response curves in Figure 1A.

For subsequent functional analyses the lower dose of 3.12 µg/mL was selected, which reduced cell viability of about 25% (Figure 1A). Cells migratory ability was assayed after 24 hours of treatment with elaborate of Agave by using wound-healing (Figure 1B-C) and transwell (Figure 1D-E) procedure, and it emerged that the elaborate of Agave hinders cell migration both in U-2 OS cell line and in Saos-2 cell line.

The greatest challenge in therapy for OS is to overcome chemoresistance. To assess whether the elaborate of Agave could improve CDDP effectiveness as apoptotic agent, the elaborate was dosed in combination with increasing doses of CDDP for 72 hours (Figure 1F-G). The elaborate of Agave sensitized OS cells to CDDP, effectively reducing EC50 (2.7- and 2,2-fold) and LC50 (3.7- and 2.7-fold) respectively for U2-OS and Saos-2 cells (Table 3 below).

	U2-OS		SAOS-2	
Samples	CDDP EC50(µM)	CDDP LC50(µM)	CDDP EC50(µM)	CDDP LC50(µM)
CTRL	5.27	13.85	9.3	13.33
Agave 3.12 µg/mL	1.94	3.79	4.3	5

Table 3: the elaborate of Agave sensitizes osteosarcoma cells to cisplatin. EC50 and LC50 for the osteosarcoma lines treated with cisplatin (CDDP) in combination with Agave (3.12 µg/mL) or EtOH control (CTRL), calculated by using Compusyn software from the dose-response curves in Figure 1F-G.

Then, clonogenic assays were performed in order to assess colony forming ability by using U2-OS and Saos-2 cell lines treated with Agave and CDDP alone or in combination (Figure 1H-I). Even though CDDP was more effective than the elaborate of agave able to reduce the numbers of colonies, the elaborate of Agave showed an effect of potentiating the inhibitory effect of CDDP on both cell lines.

The inhibitory effect of the elaborate of Agave on cell viability was also assayed in a variety of other tumour cell lines (lung, mesothelioma and breast) (Figure 8) demonstrating a consistent dose-dependent effect.

3.2 The elaborate of Agave decreases the levels of oncogenic proteins YAP and TAZ and induces apoptosis

As described in the foregoing, OS generally shows dysregulation of plural signaling pathways, among which Hippo/YAP. Also the alterations in this pathway lead to chemo- and radioresistance in OS patients. In order to clarify the antitumour mechanism of the elaborate of agave the Authors focused on alterations inside the Hippo pathway, in particular on Hippo YAP and TAZ transducers. The elaborate of Agave decreased YAP and TAZ protein expression after 24 hours in the U-2 OS cells (Figure 2A). In particular, Lats1 protein level remained unvaried, and TEAD1 proved only slightly downregulated. Reduced expression of YAP and TAZ was also observed in Saos-2, HOS and MG-63 lines after 72 hours of treatment with elaborate of Agave (Figure 2B). Instead, treatment with CDDP alone did not affect YAP and TAZ protein level (Figure 2C). Moreover, the elaborate of Agave induced apoptosis by increasing Bax protein expression, as well as Caspase 3 and PARP cleaving (lanes 1-2, Figure 2C), this effect is stronger compared to that mediated by the sole CDDP treatment (lanes 1 and 3, Figure 2C) and seems to potentiate the CDDP effect by increasing Caspase 3 cleaving in the combined treatment (lanes 3-4, Figure 2C). These data suggest that the elaborate of Agave reduces cell viability by downregulating YAP and TAZ, which are known to function as oncogens in OS cell lines. In fact, YAP/TAZ silencing sensitized OS cell lines to the elaborate of Agave (Figure 2D), reducing EC50 and LC50 concentrations respectively 2.2- and 2.2-fold (Table 4 below).

Samples	Agave EC50(µg/mL)	Agave LC50(µg/mL)
siGFP	7.6	5.2
siYAP	4	4
siTAZ	3.8	4
siYAP/siTAZ	1.8	2.4

Table 4: YAP and TAZ silencing sensitizes osteosarcoma cells to the elaborate of agave. EC_{50} and LC_{50} concentrations for the treatment with elaborate of Agave with siGFP (control), siYAP, siTAZ or siYAP/siTAZ on U-2 OS cell lines, analyzed by using Compusyn software from dose-response curves in figure 2D.

Moreover, YAP and TAZ silencing reduced the clonogenic and migratory abilities of U-2 OS and Saos-2 cell lines (Figures 2E-G and Figures 9 2A-B).

3.3. Saponins in the elaborate of Agave lower the levels of YAP and TAZ protein

The Authors have studied the effect of saponins, which are the most abundant compounds present in the natural elaborate of agave among those with recognised antitumour effects. The steroidal saponins Diosgenin, Sarsasapogenin and Solasodine were analyzed in viability assays (Figure 3A and Table 5 below), treating the cells with sublethal doses of each compound to test their effect on YAP and TAZ. Obtained data show a saponins effect on YAP and TAZ less effective than that exerted by the elaborate of Agave as such (Figure 3).

Synthetic compounds	EC50 (μM)	LC50 (μM)
Diosgenin	15.93	12.5
Sarsasapogenin	29.29	20
Solasodine	4.40	17.5

Table 5: Treatment with saponins reduces osteosarcoma cell viability. EC_{50} and LC_{50} on U-2 OS cellule treated with synthetic saponins Diosgenin, Sarsasapogenin and Solasodine, calculated by using Compusyn software from the dose-response curves in Figure 3A.

The comparison was carried out by cell viability (ATPlite) and YAP and TAZ degradation experiments. Rather than a proper comparison, the issue is of understanding which classes of compounds contained in the elaborate of Agave can at least partly sum up Agave activity.

3.4. The elaborate of Agave improves degradation of YAP and TAZ proteins as an early event

Protein stability assays were performed by treating cells with cycloheximide (CHX) in the presence or in the absence of elaborate of agave, and harvesting the cells at different times. The treatment with elaborate of Agave led to YAP and TAZ protein levels significantly reduced at 24 and 16 hours, respectively (Figure 3 C-E). YAP/TAZ reduction induced by the elaborate of Agave was mediated by ubiquitin-dependent proteasomal degradation as indicated by the increased abundance of ubiquinated

YAP/TAZ (Figure 3F-G). The rapid loss of YAP and TAZ proteins suggests that ubiquitin-mediated YAP/TAZ degradation is an early event after elaborate administration.

2.5. The elaborate of Agave reduces YAP and TAZ mRNA as a late event

In addition to the decrease of the abundance of YAP/TAZ proteins, the Authors have verified whether the elaborate of Agave would reduce YAP and TAZ mRNA expression. The treatment with elaborate of Agave for 72 hours entailed a significant decrease of YAP mRNA (2.3-fold, $p < 0.001$) and TAZ mRNA (1.9-fold, $p < 0.001$) (Figure 4A-B). This reduced YAP and TAZ ability to function as transcriptional coactivators, as the expression of their target genes: CTGF, ANKRD1 and MCM7 was also significantly downregulated (Figure 4C-E). The treatment with CDDP alone decreased YAP and TAZ expression, as well as their transcriptional function (determined by target gene expression) of 96 hours (Figure 4A-E). This is consistent with the results shown in Figure 2C, in which CDPP alone did not modulate YAP and TAZ protein levels after 72 hours of treatment. It is important to stress, however, that the effect observed was lower than the elaborate of Agave, both alone and in combination with CDDP (Figure 4A-E). TEAD1 mRNA was slightly decreased (1.2-fold, $p < 0.01$) after 72 hours of treatment with elaborate of Agave or CDDP (Figure 9), suggesting a non-specific regulation. LATS1 and LATS2 were not influenced by any treatment (Figure 9 D-E).

3.6 The elaborate of Agave downregulates YAP and TAZ mRNA by inhibiting the function of transcriptional activator NF-κB

YAP and TAZ promoter sequence for transcription factor binding sites was analyzed by using LASAGNA-Search 2.0, and plural consensus binding sites of NF-κB p65/p50 homo- and heterodimers were found inside YAP and TAZ promoters. Since the NF-κB transcription factors play an oncogenic role in various tumours and demonstrated to promote metastasis and chemoresistance in osteosarcoma, the Authors of the invention have analyzed whether the consensus association sites of NF- κB identified inside of YAP and TAZ promoters were linked to their transcriptional regulation by the elaborate of agave, by performing a ChIP analysis with NF-κB subunits p65 and p50 and histone acetylase p300 as control of transcriptionally active chromatin for the two sites (YAP) and the three sites (TAZ) provided for binding to NF-kB (Figure 5A). By using the H1H2BA as non-modulated control (Figure 5B), the Authors observed that the treatment with elaborate of Agave decreased recruitment of NF-κB p65/p50 at the first putative YAP binding site (Figure 5C) and reduced recruitment of p65, concomitantly increasing p50 binding, at the second YAP binding site (Figure 5D). The treatment with agave reduced the

recruitment of histone acetylase p300 in both YAP binding sites (Figure 5C-D), indicating an impairment of YAP transcription. Likewise, the elaborate of Agave reduced the recruitment of NF-κB p65/p50 and p300 at all three TAZ binding sites (Figure 5E-G). Of particular note, the first TAZ binding site was strongly regulated by NF-κB p65:p65 homodimers and the treatment with the elaborate of Agave abolished this recruitment (Figure 5G). Since NF-κB transcription factors bind to YAP and TAZ and regulate YAP and TAZ transcription (Figure 5), the Authors verified whether the elaborate of Agave would regulate NF-κB protein expression. The treatment of U-2 OS cells with elaborate of Agave for 48 hours produced a significant accumulation of NF-κB p50 and a slight decrease of NF-κB p65 (Figure 6A). the treatment of Saos-2, MG-63 and HOS cell lines showed a consistent expression pattern of p65 and p50 after 72 hours (Figure 6B). As described in the foregoing, NF- κB functions as dimer to activate gene transcription (p65: p65 and p65: p50) or repress it (p50: p50). Besides the total amount of the individual NF-kB subunits, the relative ratio among the different subunits determines the outcome of the NF-κB signaling. The quantitation of the ratio between the proteins p65/p50 revealed that the treatment with elaborate of Agave strongly fosters the accumulation of the inhibitory subunit p50 of NF-κB in all cell lines assayed (Figure 6C). The NF-κB dimers have to be localized in the nucleus in order to exert their transcriptional effect. The subcellular localization of NF-κB subunits after the treatment with elaborate of Agave by nuclear/cytoplasmic fractionation showed nuclear accumulation of p105 (p50 precursor) and p50, and the simultaneous reduction of p65 localization at the nucleus (Figure 6D-E). These data are consistent with the immunofluorescent staining of NF-κB p105/p50 in the absence and in the presence of IL-6 (which serves to activate NF- κB signaling) (Figure 6F-G), and of NF-κB p65 (Figure 6H-I), after the treatment with elaborate of agave. Finally, consistently with the altered expression and the localization of NF-κB subunits induced by the elaborate of agave, the expression of various NF-κB target genes was significantly reduced (Figure 6J).

CLAIMS

1. An elaborate of Agave for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

2. The elaborate for use according to claim 1, wherein said deregulation is detected in cells of said tumours with respect to healthy cells adjacent thereto.

3. The elaborate for use according to claim 1 or 2, wherein said tumours are identified by a genetic signature which includes the genes

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795

wherein, an abnormal expression of said genes in the tumour mass with respect to the healthy surrounding tissue is considered to be a genetic signature that identifies tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

4. The elaborate for use according to any one of claims 1 to 3 wherein the YAP and/or TAZ proteins show a nuclear or nucleocytoplasmic localization in the cells of said tumours.

5. The elaborate for use according to any one of claims 1 to 4, wherein said elaborate is in association with at least one chemotherapeutic agent.

6. The elaborate for use according to claim 5, wherein said chemotherapeutic agent is selected from cisplatin, iphosphamide, doxorubicin, methotrexate.

7. The elaborate for use according to any one of claims 1 to 4, wherein said elaborate is in association with at least one inhibitor of the YAP/TAZ-TEAD pathway.

8. The elaborate for use according to claim 7, wherein said inhibitor is selected from verteporfin, peptides which mimic VGLL4, dasatinib, pazopanib, dobutamine.

9. The elaborate for use according to any one of claims 1 to 8, wherein said tumours are selected from myeloid tumours, breast cancer (tumour), lung

cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer.

10. The elaborate for use according to claim 9, wherein said breast tumour is a triple negative tumour.

11. A pharmaceutical composition comprising an elaborate of agave and at least one pharmaceutically acceptable excipient and/or carrier for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

12. The composition for use according to claim 11, wherein said deregulation is detected in cells of said tumours with respect to healthy cells adjacent thereto.

13. The composition for use according to claim 11 or 12, wherein said tumours are identified by a genetic signature which includes the genes

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795

wherein, an abnormal expression of said genes in the tumour mass with respect to the healthy surrounding tissue is considered to be a genetic signature that identifies tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

14. The composition for use according to any one of claims 11 to 13, wherein the YAP and/or TAZ proteins show a nuclear or nucleocytoplasmic localization in the cells of said tumours.

15. The composition for use according to any one of claims 11 to 14, wherein said tumours are selected from myeloid tumours, breast cancer, lung cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer.

16. The composition for use according to claim 15, wherein said breast tumour is a triple negative tumour.

17. An association of an elaborate of agave, at least one chemotherapeutic agent and/or at least one inhibitor of the YAP/TAZ-TEAD pathway.

18. The association according to claim 17, wherein said chemotherapeutic agent is selected from cisplatin, Iphosphamide, doxorubicin, methotrexate.

19. The association according to claim 17 or 18, wherein said inhibitor is selected from verteporfin, peptides which mimic VGLL4, dasatinib, pazopanib, dobutamine.

20. The association according to any one of claims 17 to 19 for medical use.

21. The association according to any one of claims 17 to 20 for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

22. The association for use according to claim 21, wherein said deregulation is detected in cells of said tumours with respect to healthy cells adjacent thereto.

23. The association according to claims 21 or 22, wherein said tumours are identified by a genetic signature which includes the genes

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B ; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795

wherein, an abnormal expression of said genes in the tumour mass with respect to the healthy surrounding tissue is considered to be a genetic signature that identifies tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

24. The association for use according to any one of claims 21 to 23, wherein the YAP and/or TAZ proteins show a nuclear or nucleocytoplasmic localization in the cells of said tumours.

25. The association for use according to any one of claims 21 to 24, wherein said tumours are selected from myeloid tumours, breast cancer, lung

cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer.

26. The association for use according to claim 25, wherein said breast tumour is a triple negative tumour.

27. A pharmaceutical composition comprising an association according to any one of claims 17 to 19 and at least one pharmaceutically acceptable excipient and/or carrier.

28. The composition according to claim 27 for use in the treatment of tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

29. The composition for use according to claim 28, wherein said deregulation is detected in cells of said tumours with respect to healthy cells adjacent thereto.

30. The composition according to claims 28 or 29 wherein said tumours are identified by a genetic signature which includes the genes

AMOTL2; Angiomotin like-2	Gene ID: 51421
ANKRD1; ankyrin repeat domain 1	Gene ID: 27063
ANLN; anilin, actin binding protein	Gene ID:54443
SV2B; synaptic vesicle glycoprotein 2 B	Gene ID:9899
ALPP; alkaline phosphatase placental	Gene ID:250
SPARCL1; SPARClike1	Gene ID: 8404
KRT34; keratin34	Gene ID: 3885
STXBP6; syntaxin binding protein 6	Gene ID:29091
NAV3; neuron navigator 3	Gene ID:89795

wherein, an abnormal expression of said genes in the tumour mass with respect to the healthy surrounding tissue is considered to be a genetic signature that identifies tumours that show deregulation of the YAP and TAZ proteins and/or of the mRNA that encodes them.

31. The composition for use according to any one of claims 28 to 30, wherein the YAP and/or TAZ proteins show a nuclear or nucleocytoplasmic localization in the cells of said tumours.

32. The composition for use according to any one of claims 28 to 31, wherein said tumours are selected from myeloid tumours, breast cancer, lung cancer, liver tumour, colon cancer, pancreatic cancer, thyroid cancer, mesothelioma, osteosarcoma and ovarian cancer.

33. The composition for use according to claim 32, wherein said breast tumour is a triple negative tumour.

34. A process for the preparation of a pharmaceutical composition as defined in claim 27, wherein an agave product is combined with at least one chemotherapeutic agent and/or at least one inhibitor of the YAP/TAZ-TEAD pathway, and at least one pharmaceutically acceptable carrier and/or excipient.

5

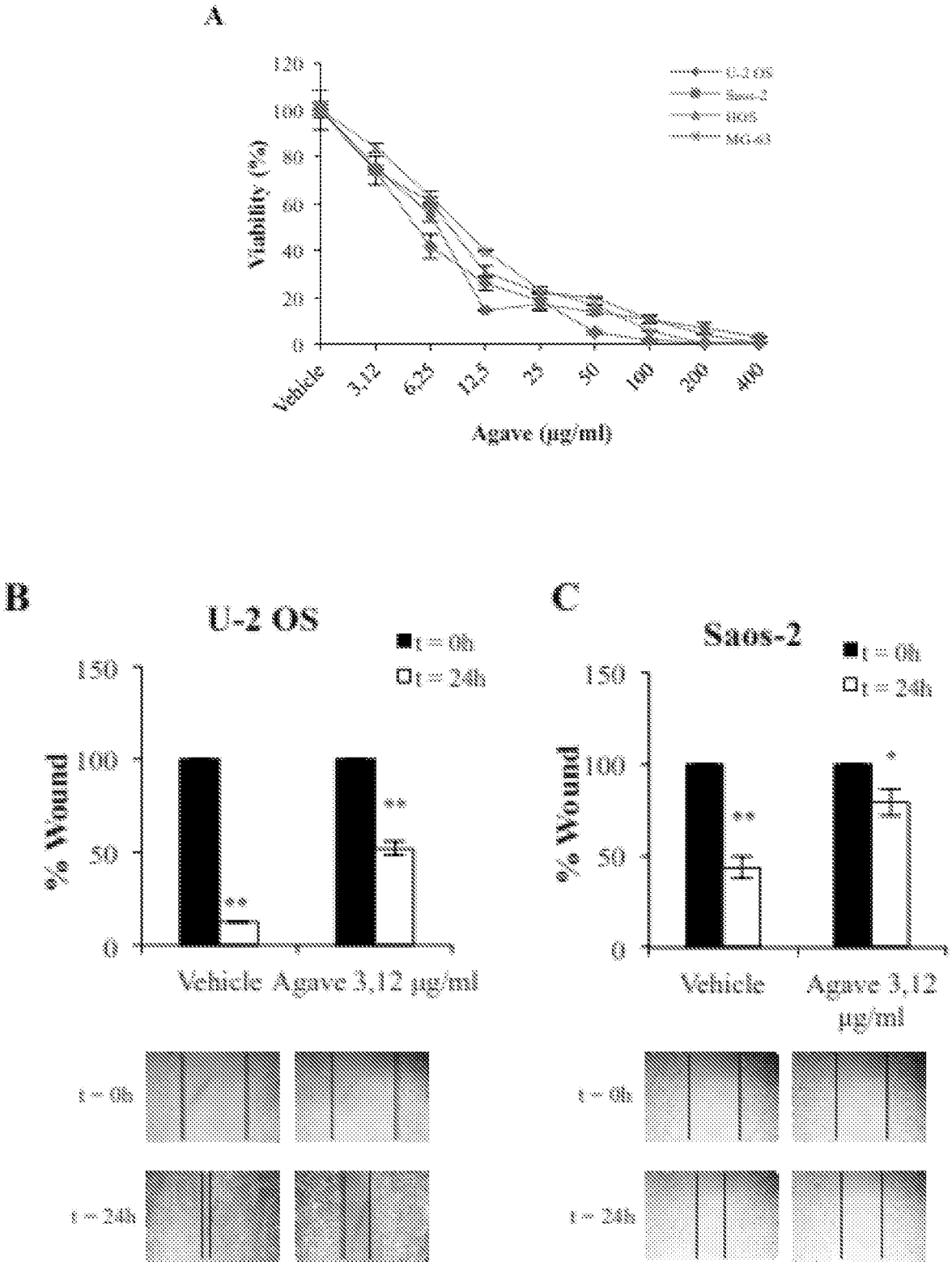

A
Viability (%)
120
100
80
60
40
20
0
Vehicle
3,12
6,25
12,5
25
50
100
200
400
Agave (µg/ml)
B
U-2 OS
t = 0h
t = 24h
% Wound
150
100
50
0
**
**
Vehicle
Agave 3,12 µg/ml
t = 0h
t = 24h
C
Saos-2
t = 0h
t = 24h
% Wound
150
100
50
0
**
*
Vehicle
Agave 3,12 µg/ml
t = 0h
t = 24h

Fig. 1 A-C

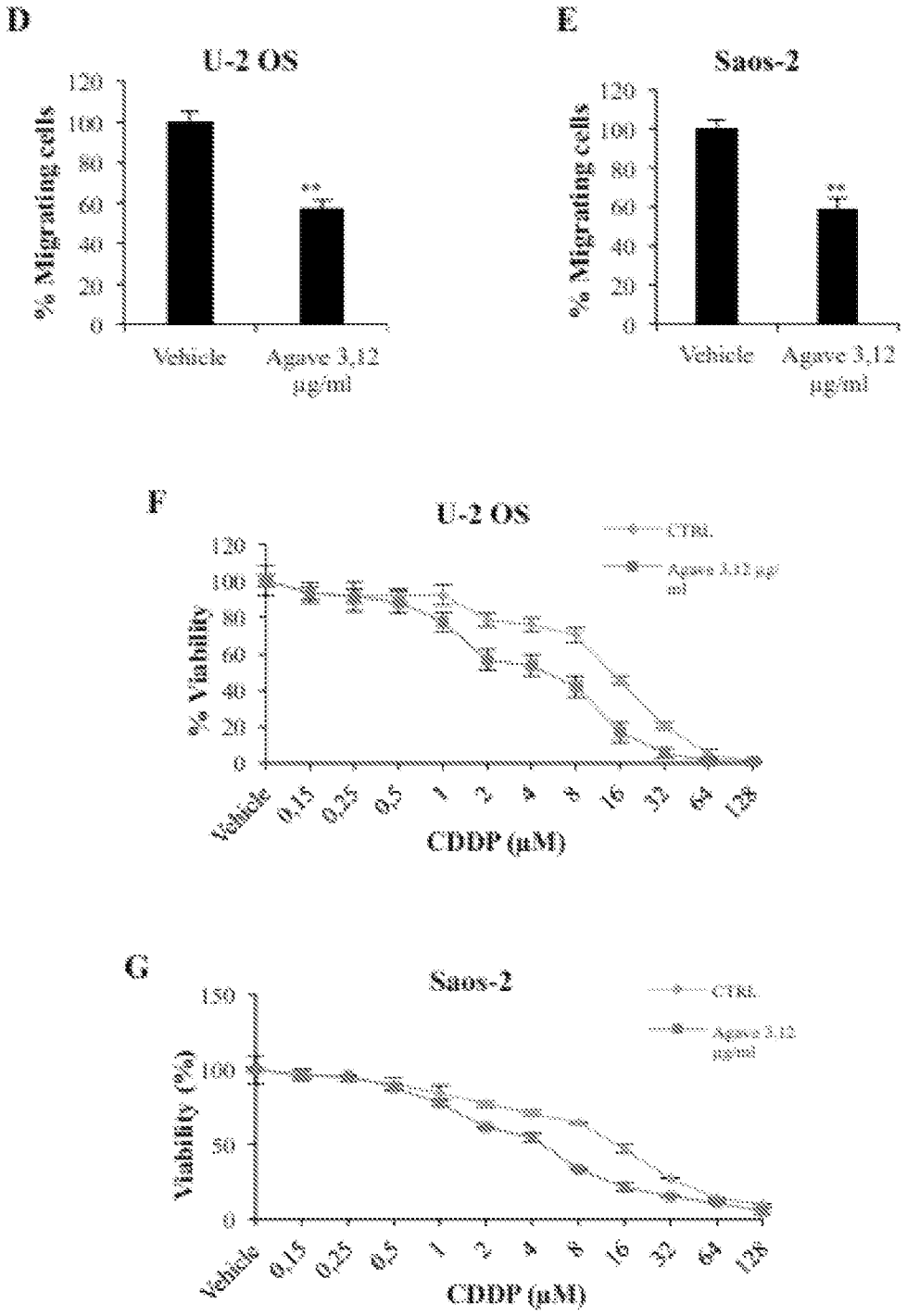
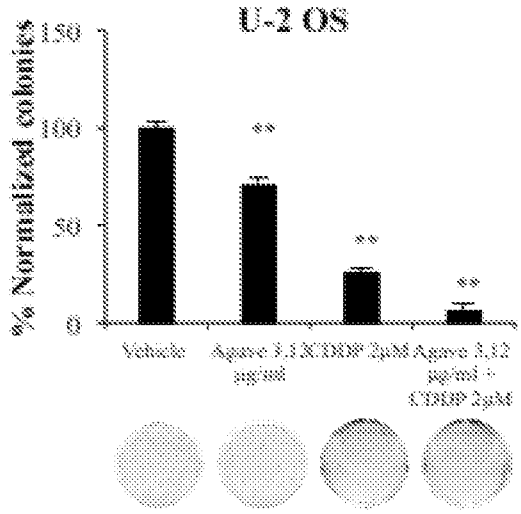

D
U-2 OS
% Migrating cells
120
100
80
60
40
20
0
Vehicle
Agave 3,12 µg/ml
E
Saos-2
% Migrating cells
Vehicle
Agave 3,12 µg/ml
F
U-2 OS
CTRL
% Viability
Vehicle
0,15
0,25
0,5
1
2
4
8
16
32
64
128
CDDP (µM)
G
Saos-2
CTRL
Viability (%)
150
100
50
0
CDDP (µM)

Fig. 1 D-G

H


U-2 OS
% Normalized colonies
150
100
50
0
**
**
**
Vehicle
Agave 3.13 µg/ml
CDDP 2µM
Agave 3.13 µg/ml + CDDP 2µM

I

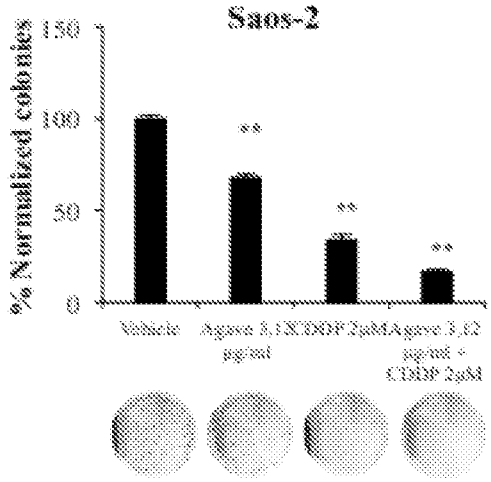

Saos-2
% Normalized colonies
150
100
50
0
**
**
**
Vehicle
Agave 3.13 µg/ml
CDDP 2µM
Agave 3.13 µg/ml + CDDP 2µM

Fig. 1 H-I

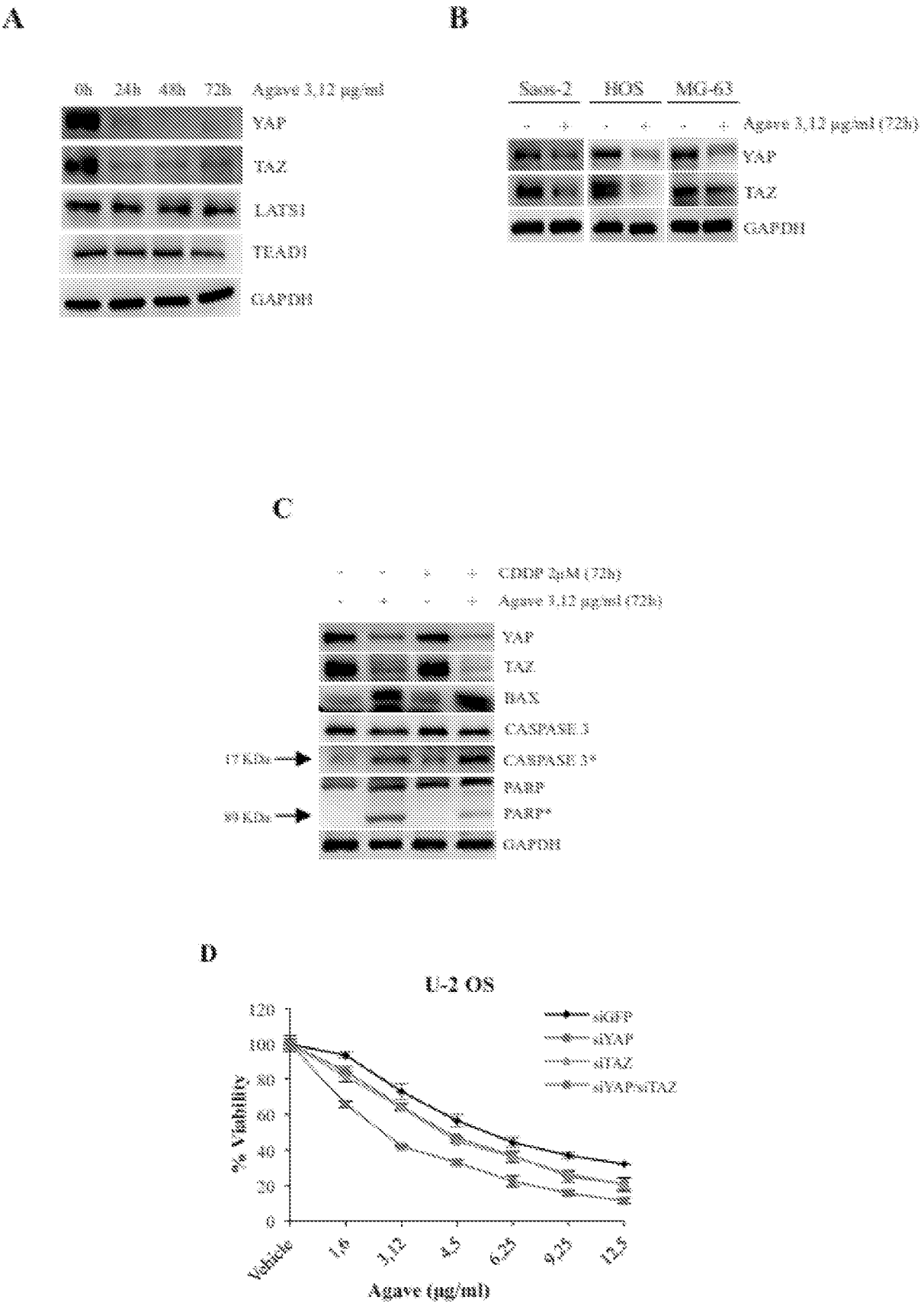
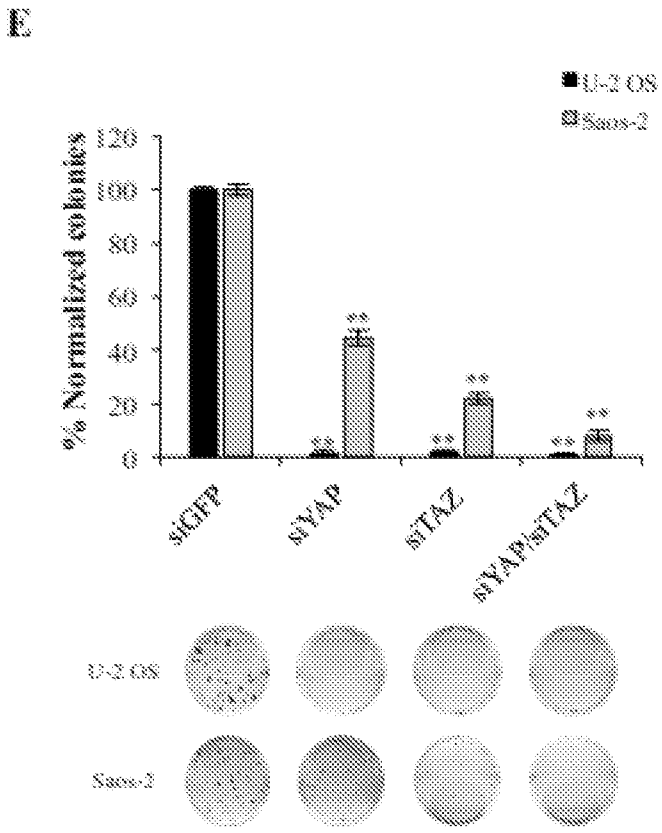

A
0h 24h 48h 72h Agave 3,12 µg/ml
YAP
TAZ
LATS1
TEAD1
GAPDH
B
Saos-2 HOS MG-63
Agave 3,12 µg/ml (72h)
YAP
TAZ
GAPDH
C
CDDP 2µM (72h)
Agave 3,12 µg/ml (72h)
YAP
TAZ
BAX
CASPASE 3
17 KDa
CASPASE 3*
PARP
89 KDa
PARP*
GAPDH
D
U-2 OS
siGFP
siYAP
siTAZ
siYAP+siTAZ
% Viability
120
100
80
60
40
20
0
Vehicle
1,6
3,12
4,5
6,25
9,25
12,5
Agave (µg/ml)

Fig. 2 A-D


E
U-2 OS
Saos-2
% Normalized colonies
120
100
80
60
40
20
0
siGFP
siYAP
siTAZ
siYAP/siTAZ
U-2 OS
Saos-2

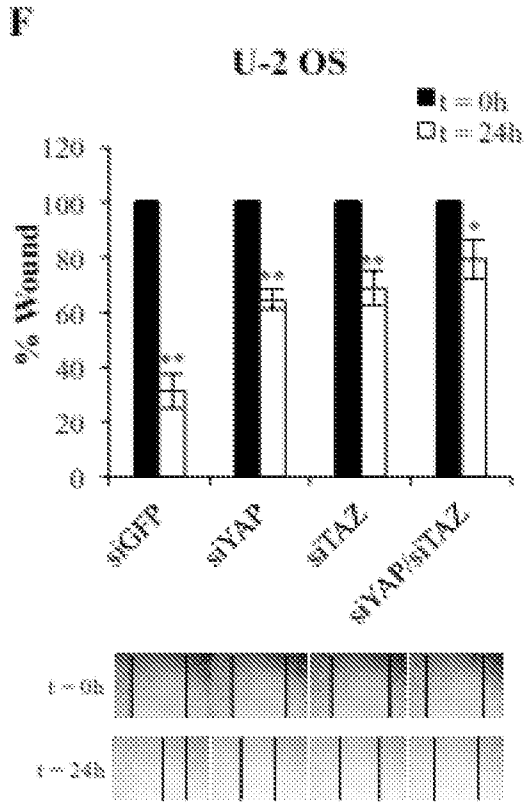

F
U-2 OS
t = 0h
t = 24h
% Wound
120
100
80
60
40
20
0
siGFP
siYAP
siTAZ
siYAP/siTAZ
t = 0h
t = 24h

Fig. 2 E-F

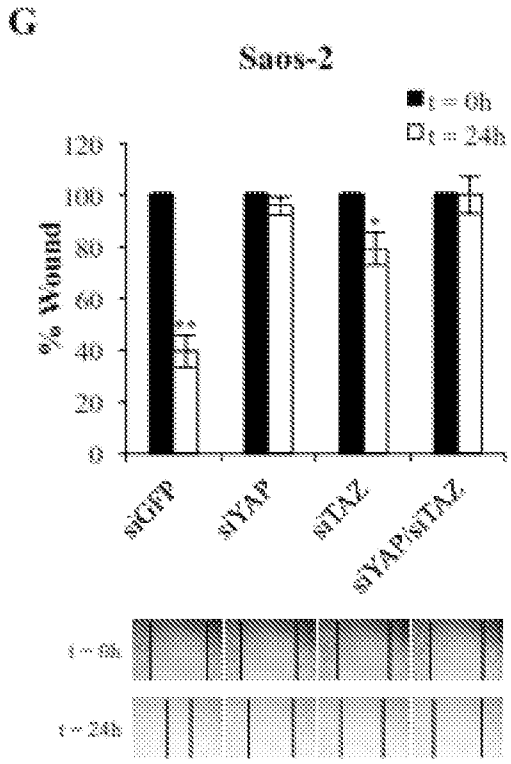


Fig. 2 G

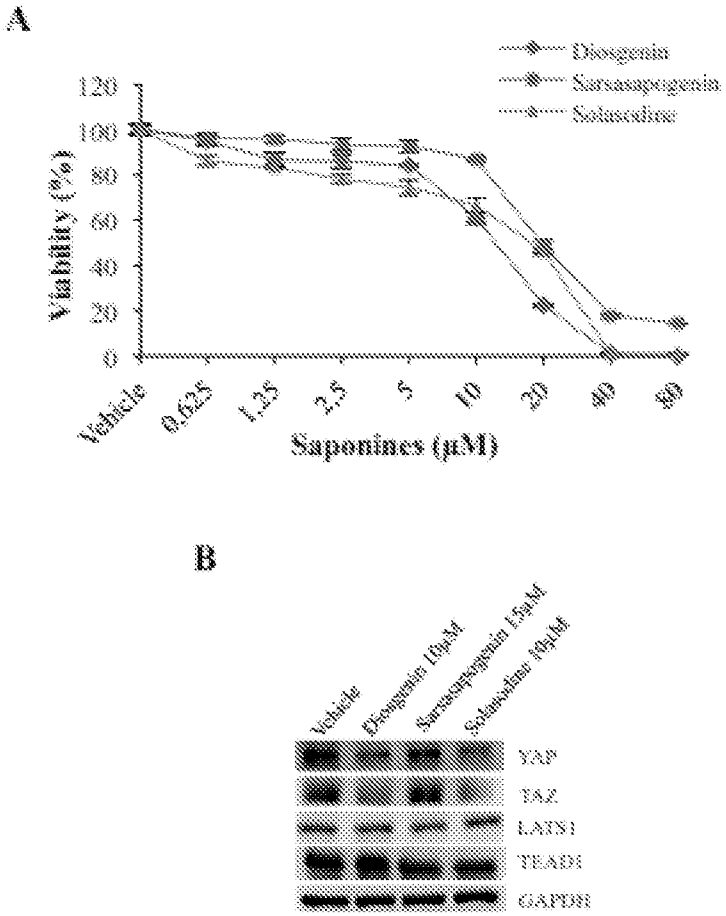


Fig. 3 A-B

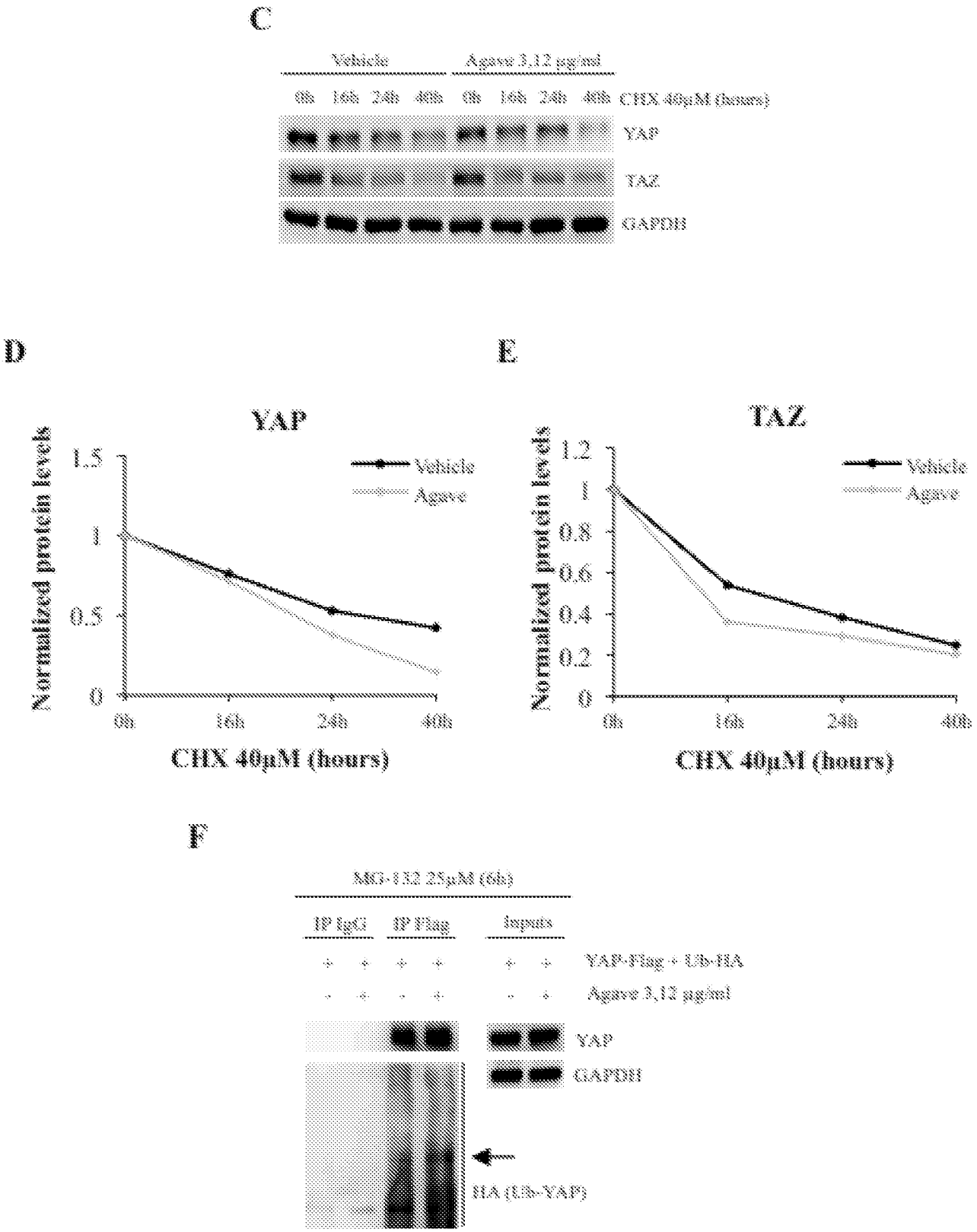

C
Vehicle
Agave 3.12 μg/ml
0h 16h 24h 40h 0h 16h 24h 40h CHX 40μM (hours)
YAP
TAZ
GAPDH
D
YAP
Normalized protein levels
1.5
1
0.5
0
Vehicle
Agave
0h 16h 24h 40h
CHX 40μM (hours)
E
TAZ
Normalized protein levels
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
Agave
0h 16h 24h 40h
CHX 40μM (hours)
F
MG-132 25μM (6h)
IP IgG
IP Flag
Inputs
YAP-Flag + Ub-HA
Agave 3.12 μg/ml
YAP
GAPDH
HA (Ub-YAP)

Fig. 3 C-F

G

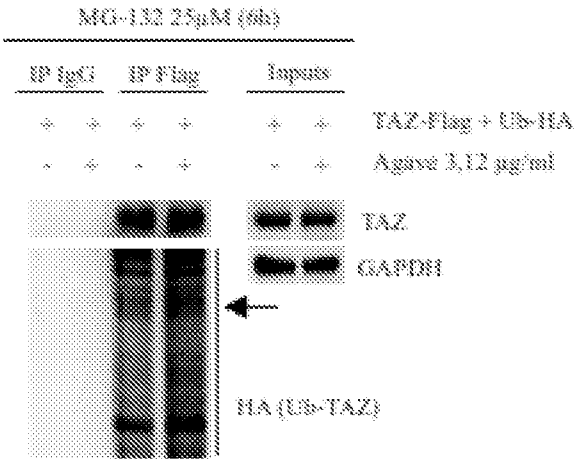
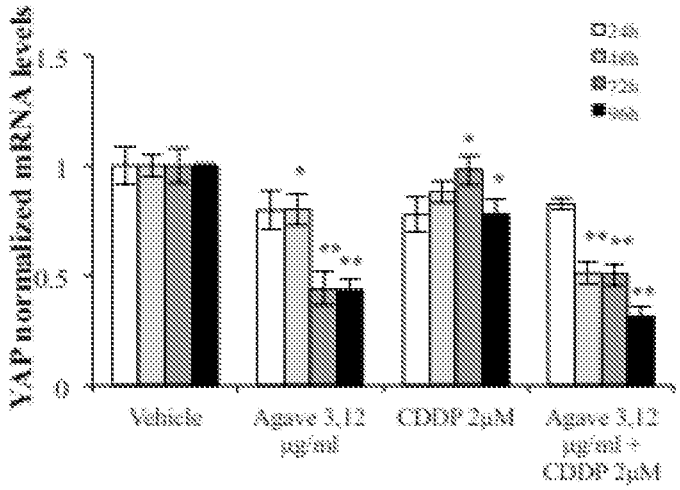


Fig. 3G

A



B

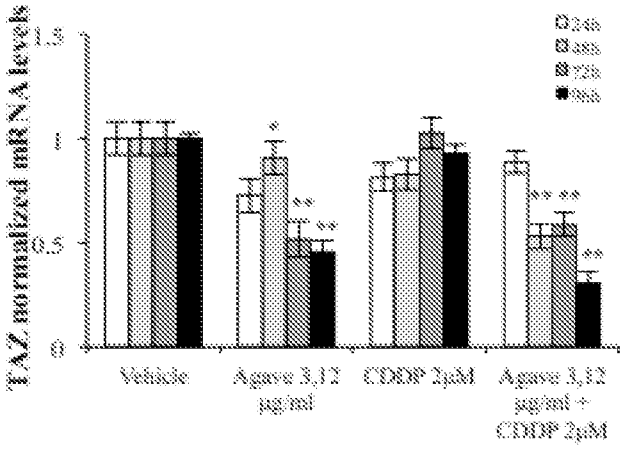
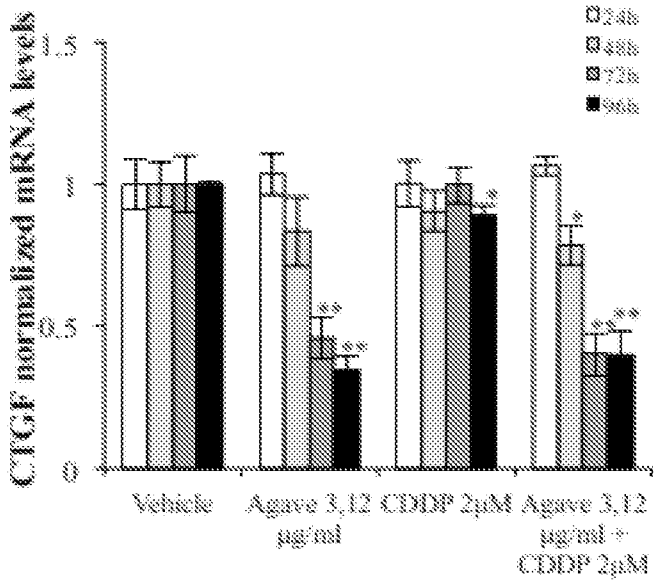


Fig. 4 A-B

C


CTGF normalized mRNA levels
1.5
1
0.5
0
24h
48h
72h
96h
Vehicle
Agave 3,12 µg/ml
CDDP 2µM
Agave 3,12 µg/ml + CDDP 2µM

D

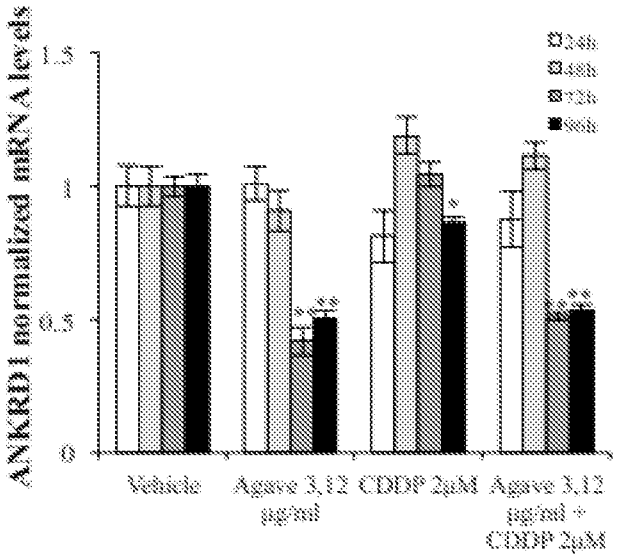

ANKRD1 normalized mRNA levels
1.5
1
0.5
0
24h
48h
72h
96h
Vehicle
Agave 3,12 µg/ml
CDDP 2µM
Agave 3,12 µg/ml + CDDP 2µM

Fig. 4 C-D

E

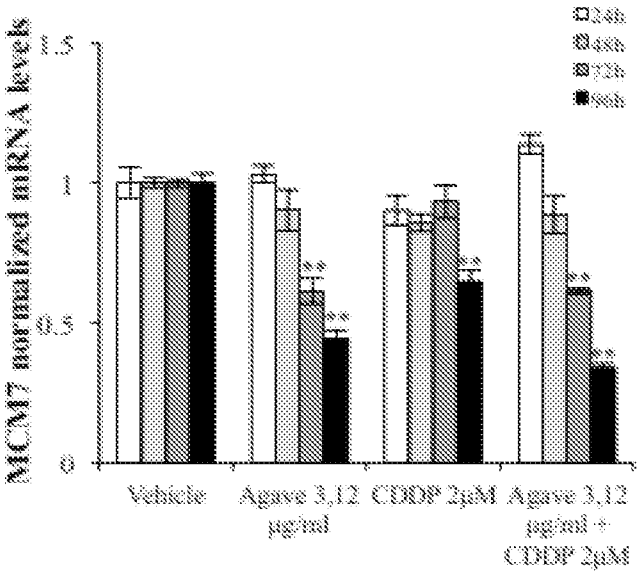
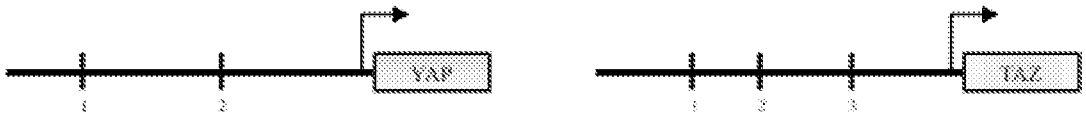


Fig. 4E

A



B

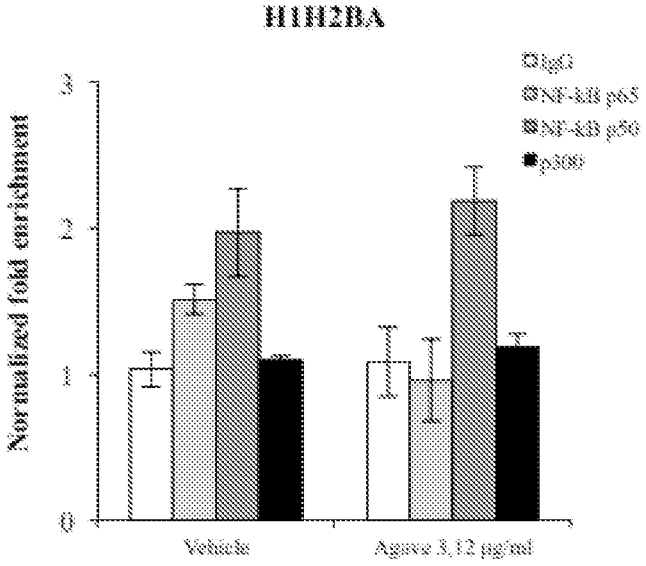
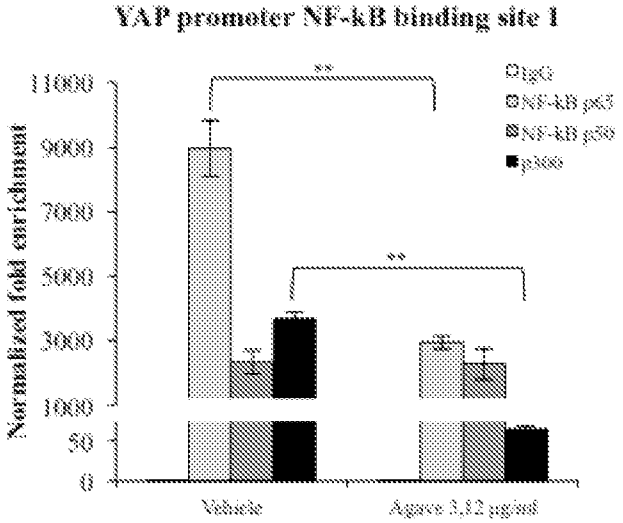
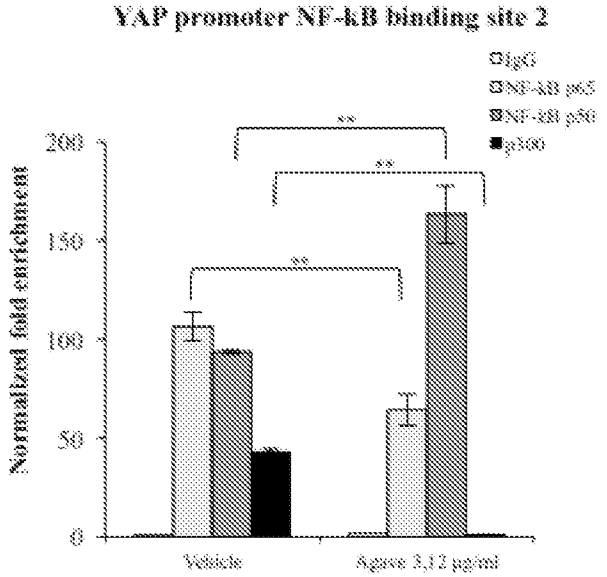


Fig. 5 A-B

C


YAP promoter NF-kB binding site 1
Normalized fold enrichment
11000
9000
7000
5000
3000
1000
50
0
**
**
IgG
NF-kB p65
NF-kB p50
p300
Vehicle
Agave 3,12 µg/ml

D


YAP promoter NF-kB binding site 2
Normalized fold enrichment
200
150
100
50
0
**
**
**
IgG
NF-kB p65
NF-kB p50
p300
Vehicle
Agave 3,12 µg/ml

E

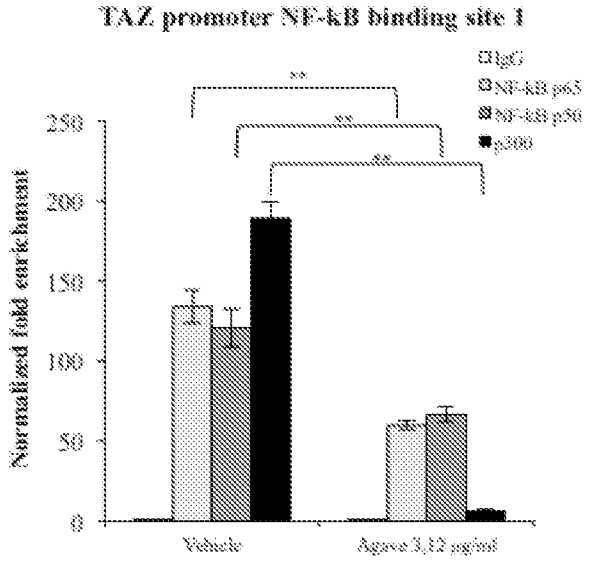
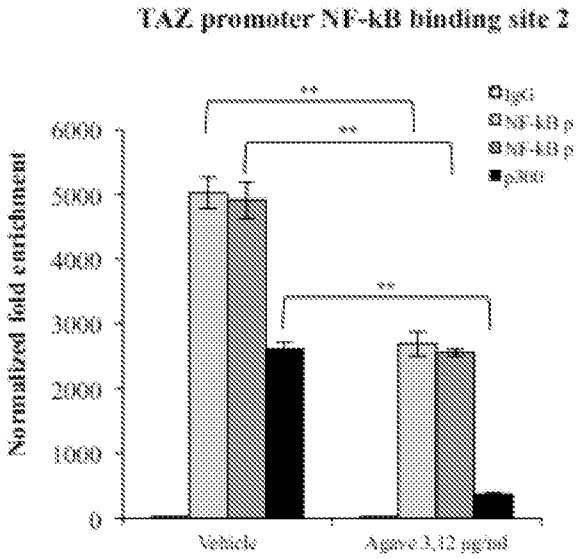

TAZ promoter NF-kB binding site 1
Normalized fold enrichment
250
200
150
100
50
0
**
**
**
IgG
NF-kB p65
NF-kB p50
p300
Vehicle
Agave 3,12 µg/ml

Fig. 5 C-E

F



G

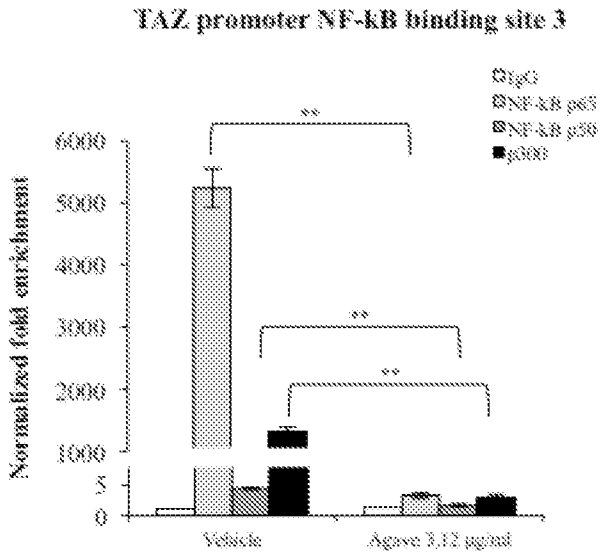
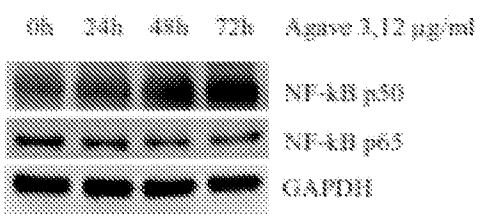


Fig. 5 F-G

A



B

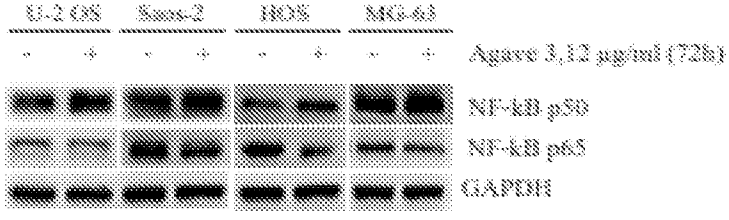


Fig. 6 A-B

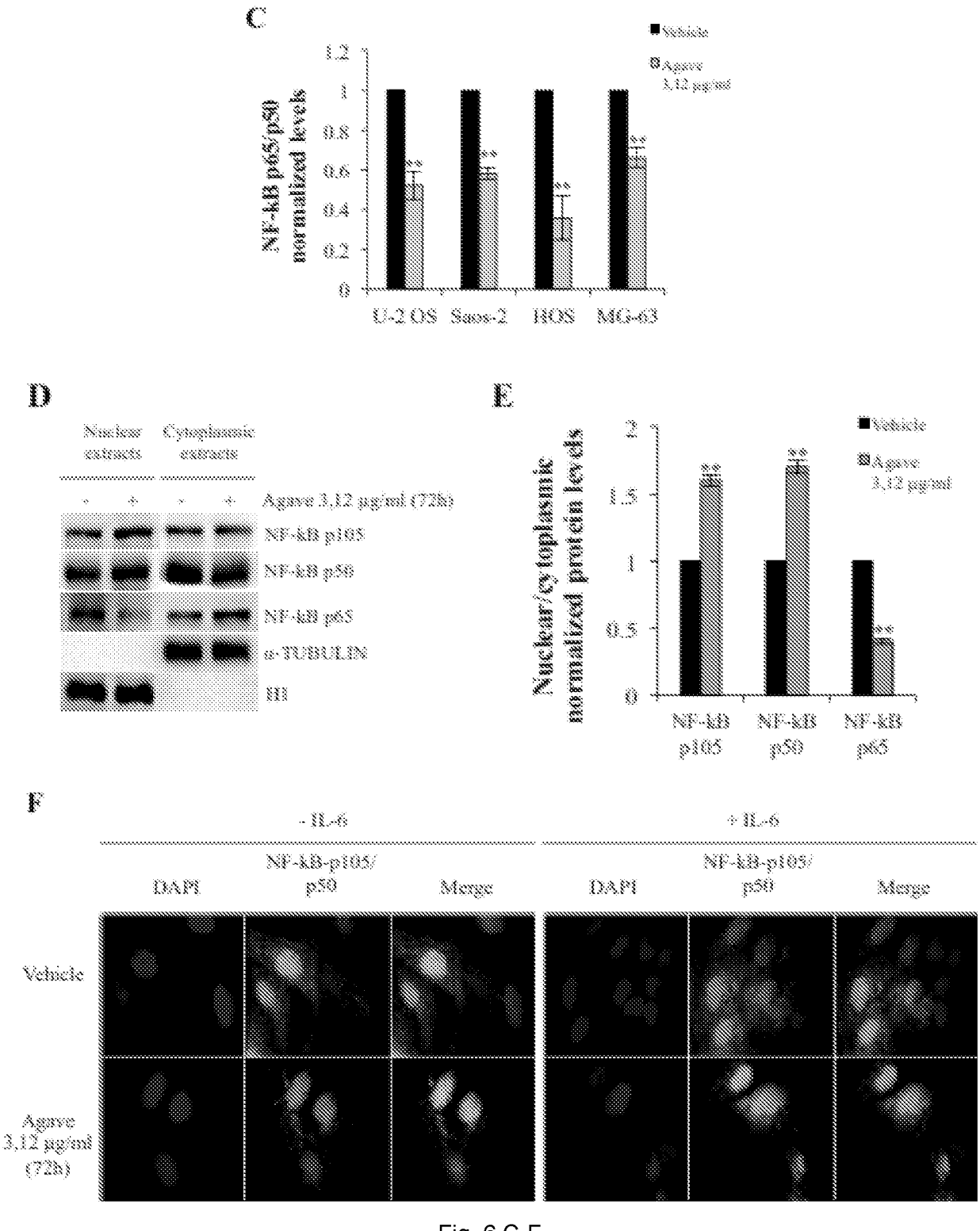

C
NF-kB p65/p50 normalized levels
Vehicle
Agave 3,12 µg/ml
U-2 OS
Saos-2
HOS
MG-63
D
Nuclear extracts
Cytoplasmic extracts
Agave 3,12 µg/ml (72h)
NF-kB p105
NF-kB p50
NF-kB p65
α-TUBULIN
HI
E
Nuclear/cytoplasmic normalized protein levels
NF-kB p105
NF-kB p50
NF-kB p65
F
- IL-6
+ IL-6
DAPI
NF-kB-p105/ p50
Merge
Vehicle
Agave 3,12 µg/ml (72h)

Fig. 6 C-F

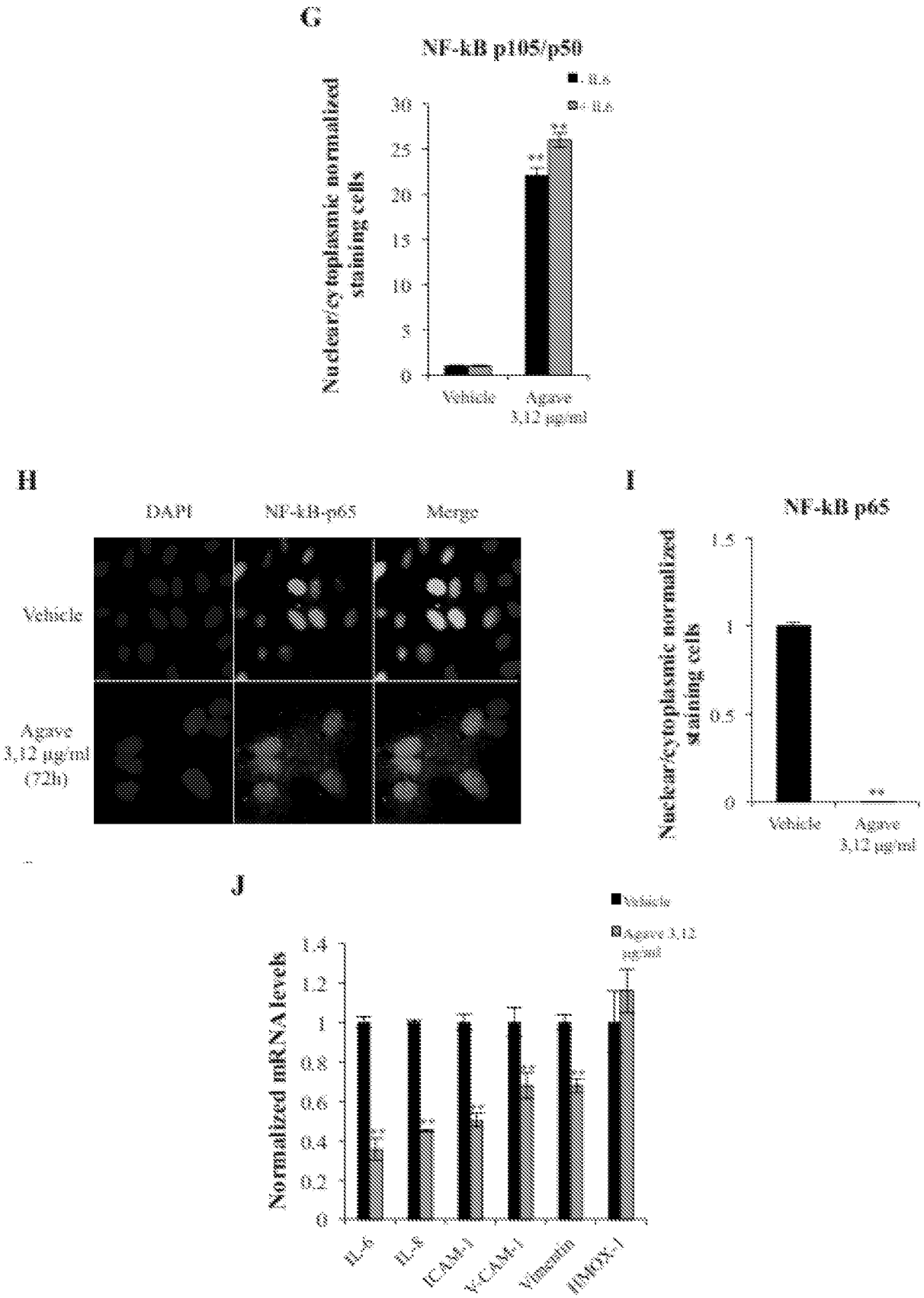

G
NF-kB p105/p50
- IL6
+ IL6
Nuclear/cytoplasmic normalized staining cells
30
25
20
15
10
5
0
Vehicle
Agave 3,12 µg/ml
H
DAPI
NF-kB-p65
Merge
Vehicle
Agave 3,12 µg/ml (72h)
I
NF-kB p65
Nuclear/cytoplasmic normalized staining cells
1.5
1
0.5
0
Vehicle
Agave 3,12 µg/ml
J
Vehicle
Agave 3,12 µg/ml
Normalized mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IL-6
IL-8
ICAM-1
V-CAM-1
Vimentin
HMOX-1

Fig. 6 G-J

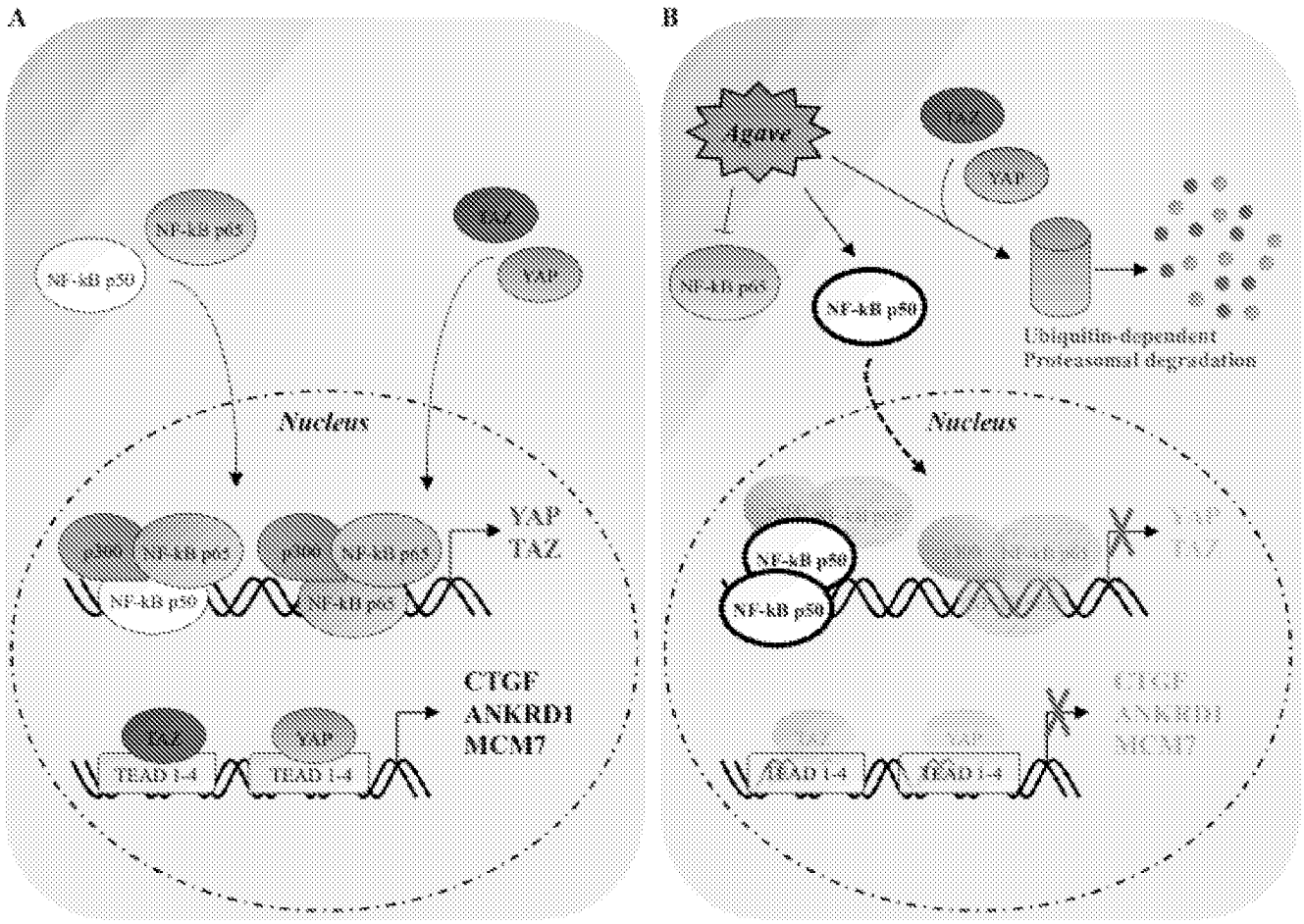

A
B
NF-kB p65
NF-kB p50
YAP
Nucleus
YAP
TAZ
NF-kB p50
NF-kB p65
CTGF
ANKRD1
MCM7
TEAD 1-4
TEAD 1-4
Agave
YAP
NF-kB p65
NF-kB p50
Ubiquitin-dependent
Proteasomal degradation
Nucleus
NF-kB p50
NF-kB p50
CTGF
ANKRD1
MCM7
TEAD 1-4
TEAD 1-4

Fig. 7

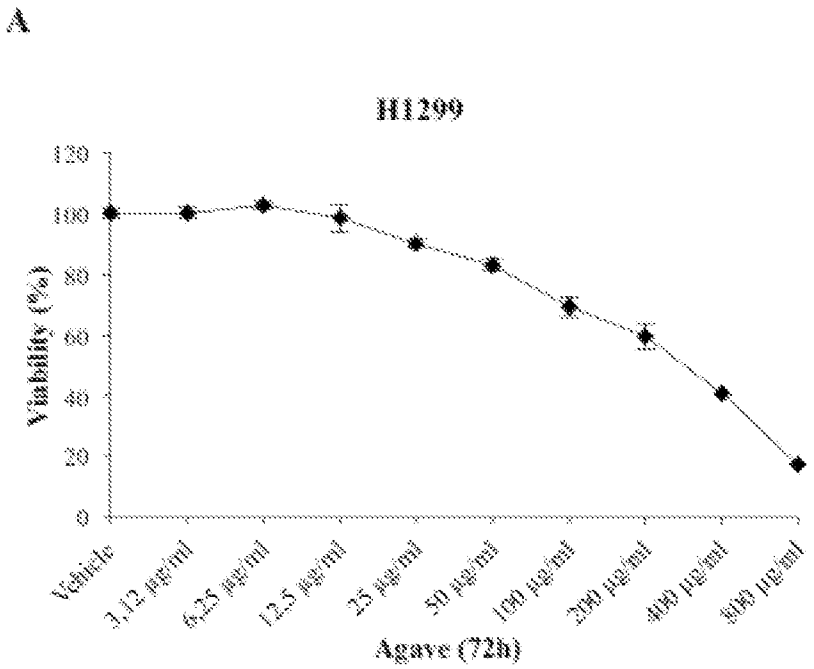
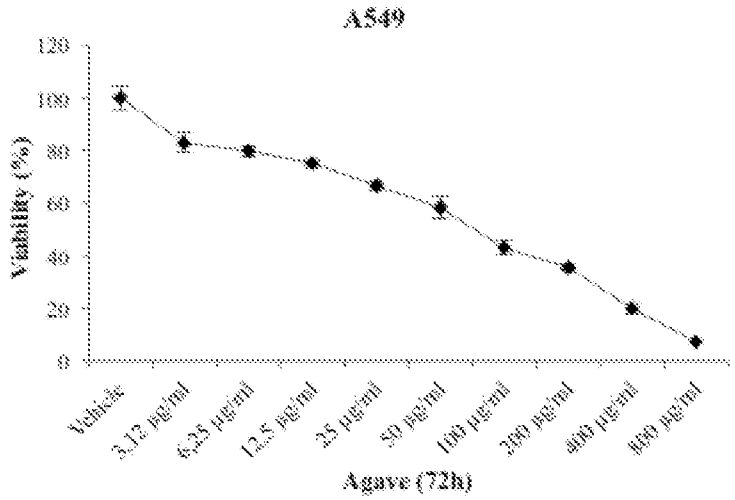
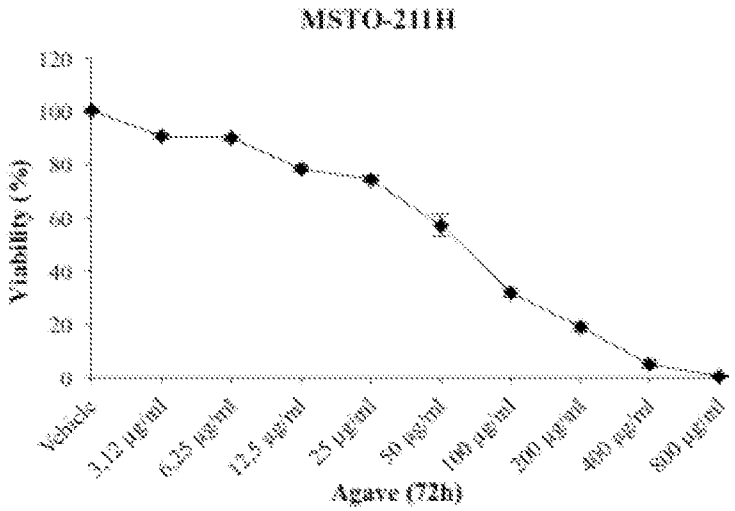

A
H1299
Viability (%)
120
100
80
60
40
20
0
Vehicle
3.12 µg/ml
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
200 µg/ml
400 µg/ml
800 µg/ml
Agave (72h)

Fig. 8 A

B


A549
Viability (%)
120
100
80
60
40
20
0
Vehicle
3.12 µg/ml
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
200 µg/ml
400 µg/ml
800 µg/ml
Agave (72h)

C


MSTO-211H
Viability (%)
120
100
80
60
40
20
0
Vehicle
3.12 µg/ml
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
200 µg/ml
400 µg/ml
800 µg/ml
Agave (72h)

D

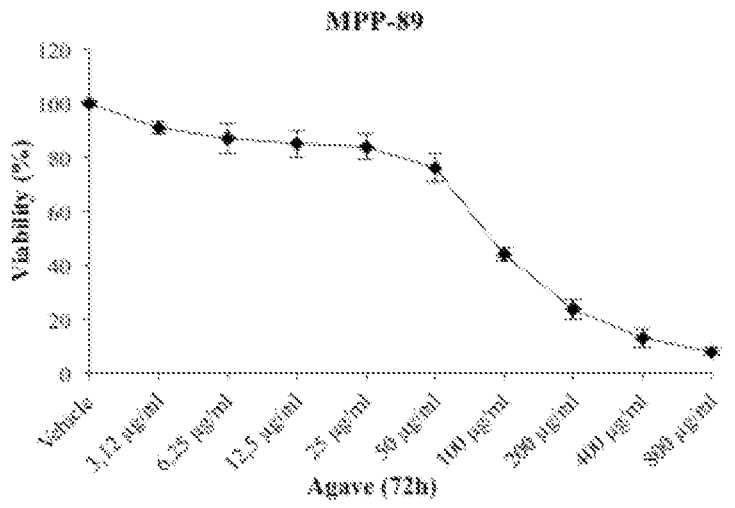
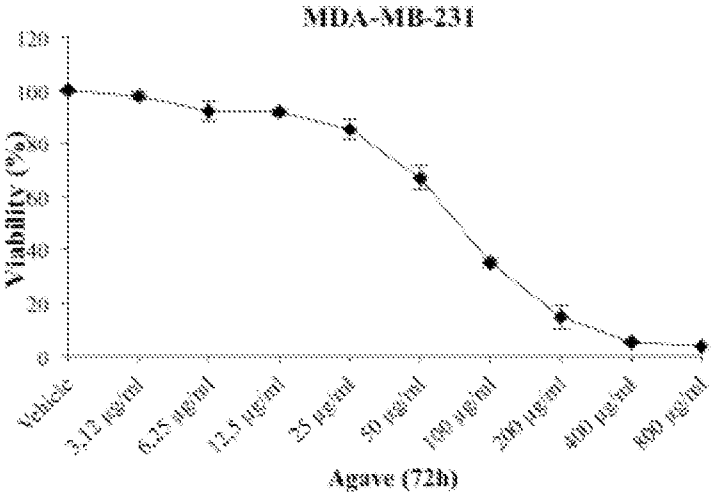

MPP-89
Viability (%)
120
100
80
60
40
20
0
Vehicle
3.12 µg/ml
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
200 µg/ml
400 µg/ml
800 µg/ml
Agave (72h)

Fig. 8 B-D

E



F

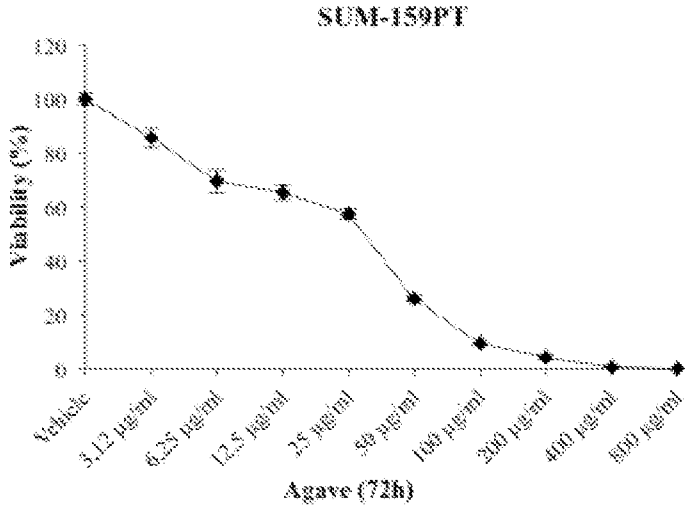


Fig. 8 E-F

A

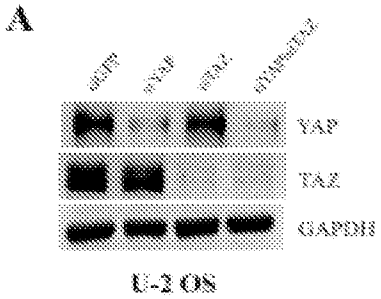


Fig. 9 A

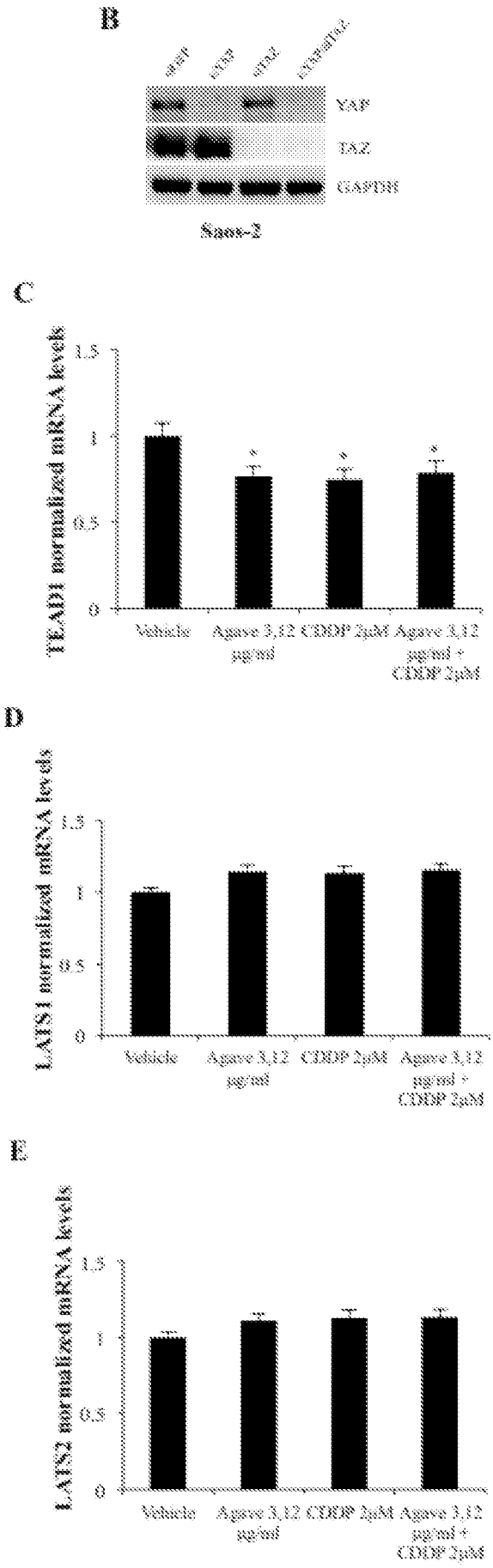

B
siCT
siYAP
siTAZ
siYAP+siTAZ
YAP
TAZ
GAPDH
Saos-2
C
TEAD1 normalized mRNA levels
1.5
1
0.5
0
Vehicle
Agave 3,12 µg/ml
CDDP 2µM
Agave 3,12 µg/ml + CDDP 2µM
D
LATS1 normalized mRNA levels
1.5
1
0.5
0
Vehicle
Agave 3,12 µg/ml
CDDP 2µM
Agave 3,12 µg/ml + CDDP 2µM
E
LATS2 normalized mRNA levels
1.5
1
0.5
0
Vehicle
Agave 3,12 µg/ml
CDDP 2µM
Agave 3,12 µg/ml + CDDP 2µM

Fig. 9 B-E

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/051762

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/88 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SANTOS-ZEA LILIANA ET AL: "Fast Centrifugal Partition Chromatography Fractionation of Concentrated Agave (Agave salmiana) Sap to Obtain Saponins with Apoptotic Effect on Colon Cancer Cells", PLANTS FOODS FOR HUMAN NUTRITION, KLUWER ACADEMIC PUBLISHERS, NL, vol. 71, no. 1, 23 December 2015 (2015-12-23), pages 57-63, XP035929347, ISSN: 0921-9668, DOI: 10.1007/S11130-015-0525-2 [retrieved on 2015-12-23]	1-4,9-15
Y	the whole document	1-34

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2019	02/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Thalmair, Michaela

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	QIN Y ET AL: "Medicine used for, e.g. treating liver cancer, includes lucid ganoderma, Antrodia camphorate, elecampane, emblic leafflower fruit, Meconopsis, Agave angustifolia, Urena lobata, tabasheer, and Herpetospermum pedunculosum seed", WPI / 2017 CLARIVATE ANALYTICS,, vol. 2011, no. 80, 21 September 2011 (2011-09-21), XP002785566,	1-4,9-15
Y	abstract	1-34

X	CHU L ET AL: "Traditional Chinese medicine composition used e.g. for treating lung cancer, includes Paris polyphylla, herba scutellariae barbatae, fiveleaf gynostemma herb, Cephalotaxus oliveri, Indian iphigenia bulb, and Actinidia and Macaranga tanarius", WPI / 2017 CLARIVATE ANALYTICS,, vol. 2015, no. 56, 20 May 2015 (2015-05-20), XP002785567,	1-4,9-15
Y	abstract	1-34

X	CHEN P Y ET AL: "Cytotoxic steroidal saponins from Agave sisalana", PLANTA MEDICA, THIEME VERLAG, DE, 1 May 2011 (2011-05-01), pages 929-933, XP018500106, ISSN: 0032-0943, DOI: 10.1055/S-0030-1250672	1-4,9-15
Y	abstract	1-34

X	RIVERA-HUERTA MARISOL ET AL: "Functional Effects of Prebiotic Fructans in ColonCancer and Calcium Metabolism in Animal Models", BIOMED RESEARCH INTERNATIONAL, HINDAWI PUBLISHING CORPORATION , vol. 2017 15 February 2017 (2017-02-15), 15 February 2017 (2017-02-15), pages 1-10, XP009508566, ISSN: 2314-6133, DOI: 10.1155/2017/9758982 Retrieved from the Internet: URL:http://downloads.hindawi.com/journals/bmri/2017/9758982.xml [retrieved on 2017-02-15]	1-4,9-15
Y	the whole document	1-34

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE OLIVEIRA JOÃO VICTOR A ET AL: "Saponin-rich fraction from Agave sisalana: effect against malignant astrocytic cells and its chemical characterisation by ESI-MS/MS", NATURAL PRODUCT RESEARCH, TAYLOR & FRANCIS, LONDON, 2 February 2018 (2018-02-02), pages 1-4, XP009508549, ISSN: 1478-6427, DOI: 10.1080/14786419.2018.1434633	1-4,9-15
Y	the whole document	1-34

X	BIANCHI E ET AL: "Antitumor agents from Agave schottii (Amaryllidaceae)", JOURNAL OF PHARMACEUTICAL SCIENCES, AMERICAN PHARMACEUTICAL ASSOCIATION, US, vol. 58, no. 5, 30 April 1969 (1969-04-30) , pages 589-591, XP009508561, ISSN: 0022-3549, DOI: 10.1002/JPS.2600580516	1-4,9-15
Y	the whole document	1-34

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Y	ZHANG WEN-QIAN ET AL: "Targeting YAP in malignant pleural mesothelioma", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, UNIVERSITY PRESS CAROL DAVILA, BUCHAREST, RO , vol. 21, no. 11 1 November 2017 (2017-11-01), pages 2663-2676, XP009508559, ISSN: 1582-1838, DOI: 10.1111/JCMM.13182 Retrieved from the Internet: URL:https://api.wiley.com/onlinelibrary/tdm/v1/articles/10.1111%2Fjcmm.13182 [retrieved on 2017-05-04] the whole document	1-34

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2019/051762

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2017319648 A1	09-11-2017	CN 105524139 A	27-04-2016
		EP 3208280 A1	23-08-2017
		US 2017319648 A1	09-11-2017
		WO 2016058547 A1	21-04-2016