BIOCHEMICAL EVALUATIONS OF Zingiberaceae sp. AND TRANSCRIPTOMICS PROFILING OF UV-IRRADIATED NORMAL HUMAN ADULT DERMAL FIBROBLAST CELLS FOR ANTI-AGING

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By

ALAFIATAYO AKINOLA ADEKOYA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

To my late Parents

Mr. Gabriel Babatunde Alafiatayo

And

Mrs. Ebunlomo Alice Alafiatayo

May your gently Souls continue to Rest in bosom of the Lord

“Education is the most powerful weapon which you can use to change the world”
-Nelson Mandela
Skin aging is the gradual building up of molecular damages as a result of production of reactive oxygen species (ROS) due to the vulnerability of the skin to external damaging factors such as solar ultraviolet (UV) radiation. The desire to look youthful is increasingly growing among men and women in today’s world and this has resulted to people willing to spend fortune on anti-aging cosmetic products. However, one major set-back in fighting premature skin aging is that, the numerous anti-aging products currently flooding the markets lack proven efficacy and they have also been reported to be toxic to the human skin. Hence, there is a need to develop an anti-aging cosmetics product from a natural product source with scientifically proven efficacy without any negative side effects. Therapeutic approach to the management of skin aging is to induce the proliferation of dermal fibroblast cells for the production of procollagen and subsequent inhibition of extracellular degrading enzymes (ECM). Plants are source of precursors of many natural products and secondary metabolites with pharmacological and therapeutic potentials. Zingiberaceae family is plants species endowed with great antioxidative properties and are widely distributed in the tropics especially Southeast Asia. The main objective of this study is to evaluate 10 selected indigenous Zingiberaceae plants for their anti-wrinkle potentials via the proliferation of UV irradiated normal human adult fibroblast cells. The selected Zingiberaceae rhizomes were screened by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant potential (FRAP) methods for total antioxidant capacity and with high performance liquid chromatography (HPLC) for flavonoid identification and quantification. Biochemical profile was investigated with total protein, lipid, total hydrolysable and reducing sugar, beta carotene and ascorbic acids assays. The profiling of fatty acid was performed using GC-FID fatty acids methyl esters method. Based on the preliminary screening, C. xanthorrhiza and C. longa showed the most potent extracts and were selected for further evaluation. The proliferating capacity of extract on normal human adult dermal fibroblast cells was
determined using 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay and anti-wrinkle potentials was assessed by the ability of extracts to inhibit the degrading enzymes, while in vivo toxicity assessment was evaluated with embryos and larval of Zebrafish. Detail molecular profiling was carried out using RNA sequencing technology for the determination of differential expressed genes (DEG). Results obtained from bioinformatics analysis were subjected to Real-Time qPCR. The results revealed high antioxidant capacity in methanol extract of C. xanthorrhiza and C. longa of 245.40±0.5 mg TE/g FW and 270.40±1.6 mg TE/g FW, respectively and were significant at (p<0.05) than other solvents. Although there were variations in different biochemical compounds, C. xanthorrhiza was found to be the topmost of all with 0.52 mg/g FW, 0.1 mg/g FW, 716.73 μg β-carotene/mg FW, 8.7% and 67.05 μg ascorbic/mg FW, respectively. GC-FID fatty acid methyl ester profile revealed the presence of both saturated and unsaturated in most of the samples while C. xanthorrhiza was found to contain more linoleic fatty acid in its oil hence conferred as an excellent candidate for anti-aging cream formulation. C. xanthorrhiza was found to be the best inhibitor of collagenase and hyaluronidase activity with 71.33% and 49.78%, respectively while Z. zerumbet displayed the highest elastase inhibition with 87.24% inhibition. Furthermore, extracts from both C. longa and C. xanthorrhiza promoted the proliferations of UV irradiated fibroblast cells at post extract treatment with percentage cell proliferation of 117.4% and 136.1%, respectively, relative to the control. The toxicity assessment of both extracts were found to be embryotoxic with similar teratogenic effects on the Zebrafish embryos and larvae at concentration above 62.5 μg/mL exposed for five days. Based on the therapeutic index (TI) calculated for five days (1.02, 1.00, 1.01, 1.12 and 1.14). C. xanthorrhiza extract was discovered less toxic, therefore was selected for molecular study. The RNA-Sequencing produced about 80 million reads in both UV irradiated and UV irradiated treated samples and 2007 genes were found to be up-regulated and 2791 genes down regulated in UV-irradiated human dermal fibroblast (HDF) cells (Sample U1). In the same manner, extract of C. xanthorrhiza treated UV- irradiated HDF cells (Sample T2) yielded 2284 up-regulated genes and 2968 down regulated genes while the comparison of the two results generated 19 genes up regulated and 19 genes down regulated these set of genes were the target genes in this study. About 19000 transcripts were reported as novel and gene ontology (GO) functional annotations have categorized the genes into various functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis implicated cancer and cytokine-cytokine receptor interaction in UV-irradiated HDF cells leading to induction of cells apoptosis as reported in the cell proliferation results in this study. The Real-Time qPCR gene expression profiling confirmed the expression of eight significantly differential expressed genes that were selected from the list of target genes to be in the same trend as obtained in the RNA-Seq analysis. HIST1H2AG, ELOVL3, OSR2 and TNFSF10 were up-regulated in sample U1 but down regulated in sample T2 while FAM111B, IVL, MFSD2A and CCNE2 were down-regulated in sample U1 but Up-regulated in sample T2. Thus, these set of confirmed genes were concluded to be potential candidates’ for biomarkers development for diagnostic, personalize and precise treatment of UV-induced premature aging. Therefore, C. xanthorrhiza could be potential lead to address the problems and issues of toxicity and efficacy associated with most available anti-aging cream.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENILAIAN BIOKIMIA Zingiberaceae sp. dan PROFIL TRANSKRIPTOMIK SEL FIBROBLAS DERMAL MANUSIA DEWASA NORMAL TERADIASI-UV UNTUK KESAN ANTI-PENUAAN

Oleh

ALAFIATAYO AKINOLA ADEKOYA

Mei 2017

Pengerusi : Noor Azmi Shaharuddin, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

untuk analisis lanjut. Keupayaan ekstrak ini dalam proliferasi sel fibroblas telah ditentukan dengan menggunakan asai MTT dan potensi anti-kedut telah ditentukan oleh keupayaan ekstrak untuk menghalangi fungsi enzim degradasi ECM (elastase, hyaluronidase dan kolagenase), manakala kesan toksisiti in vivo dinilai menggunakan embrio dan larva ikan Zebra (Danio rerio). Profil molekul telah dijalankan dengan menggunakan penjujukan RNA untuk menentukan perbezaan gen terekspressi (DEG). Keputusan yang diperolehi daripada analisis bioinformatik disahkan secara analisis qPCR. Keputusan mendapati kapasiti antioksidasi yang tinggi dalam ekstrak metanol oleh C. xanthorrhiza dan C. longa dengan 245.40 ± 0.5 mg TE/g dan 270.40 ± 1.6 mg TE/g, masing-masing adalah signifikan pada (p <0.05) berbanding pelarut lain. Walaupun terdapat variasi dalam sebatian biokimia, C. xanthorrhiza didapati paling tinggi dalam semua analisis, iaitu 0.52 mg/g FW, 0.1 mg/g FW, 716.73 μg β-karotena/mg FW, 8.7% dan 67.05 μg askorbik/mg FW, masing-masing. Analisis profil asid lemak metil ester menggunakan GC-FID mendedahkan kehadiran kedua-dua asid lemak tepu dan tak tepu dalam kebanyakkan sampel, manakala C. xanthorrhiza didapati mengandungi asid lemak linoleik yang tinggi dalam minyaknya, dan ia sesuai untuk dijadikan formulasi krim anti-penuaan. C. xanthorrhiza didapati menghalangi aktiviti collagenase dan hyaluronidase tertinggi dengan 71.33% dan 49.78% manakala Z. zerumbet merencatkan elastase tertinggi. Tambahana pula, kedua-dua ekstrak C. longa dan C. xanthorrhiza didapati meningkatkan proliferasi sel fibroblas yang di UV radiasi selepas rawatan ekstrak dengan peratusan pertumbuhan sel sebanyak 117.4% dan 136.1%, masing-masing berbanding dengan kawalan. Penilaian ketoksitisan kedua-dua ekstrak didapati embriotoksik dengan kesan teratogenik yang sama pada embrio dan larva Zebrafish di kepekatan melebihi 62.5 μg/mL selepas terdedah selama lima hari. Ekstrak C. xanthorrhiza kurang toksik dan meningkatkan proliferasi sel fibroblas dan dipilih untuk kajian lanjut di peringkat molekul. Penjujukan RNA menghasilkan kira-kira 80 juta bacaan dalam kedua-dua sampel UV dan UV terawat, 2007 gen telah didapati terekspress dan 2791 gen telah direncat dalam sel-sel kulit manusia (HDF) UV-radiasi (Sampel U1). Dengan cara yang sama, ekstrak C. xanthorrhiza sel HDF UV-radiasi terawat (Sampel U2) menghasilkan 2284 gen terekspress dan 2968 gen terekspress dalam sampel UV terawat. 2768 gen terekspress pada sampel UV dan UV terawat, 19 gen terekspress dan 19 gen terencat tersebut adalah gen sasaran dalam kajian ini. Kira-kira 19000 transkrip dilaporkan sebagai novel dan analisis ontologi gen telah mengklasifikasikan gen-gen kepada pelbagai fungsi. Analisis KEGG telah mencadangkan pembabitan kanser dan interaksi reseptor sitokin-sitokin dalam sel-sel HDF UV-radiasi yang membawa kepada induksi sel apoptosis seperti yang dilaporkan dalam hasil proliferasi sel dalam kajian ini. Profil gen ekspresi secara qPCR mengesahkan lapan gen terekspress secara ketara yang telah dipilih dari senarai gen sasaran untuk berada dalam turutan yang sama seperti yang diperolehi dalam analisis NGS. HIST1H2AG, ELOVL3, OSR2 dan TNFSF10 telah terekspress dalam sampel U1 dan terencat dalam sampel T2 manakala FAM111B, IVL, MFSD2A dan CCNE2 terekspress dalam sampel U1 dan terencat dalam sampel T2. Oleh itu, set gen-gen ini disahkan sesuai untuk menjadi calon yang baik dalam pembangunan penanda biologi bagi tujuan diagnostic dan rawatan personalisasi yang disebabkan oleh penuaan pra-matang akibat induksi UV. Oleh itu, C. xanthorrhiza berpotensi untuk menangkan masalah dan isu ketoksikan dan keberkesanan yang berkaitan dengan krim anti-penuaan.
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I certify that a Thesis Examination Committee has met on 18 May 2017 to conduct the final examination of Alafiatayo Akinola Adekoya on his thesis entitled "Biochemical Evaluations of Zingiberaceae sp. and Transcriptomics Profiling of UV-Irradiated Normal Human Adult Dermal Fibroblast Cells for Anti-Aging" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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<tr>
<td>μL</td>
<td>Micro litre</td>
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<tr>
<td>μM</td>
<td>Micromola</td>
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<tr>
<td>AP-1</td>
<td>Activated protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl</td>
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<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<tr>
<td>F.A</td>
<td>Fatty acid</td>
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<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
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<tr>
<td>FAO</td>
<td>Food and Agricultural organization</td>
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<td>FRAP</td>
<td>Ferric reducing antioxidant potential</td>
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<tr>
<td>FW</td>
<td>Fresh weight</td>
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<tr>
<td>GAE</td>
<td>Gallic equivalent</td>
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<td>GC-FID</td>
<td>Gas chromatography flame ionization detector</td>
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<td>GO</td>
<td>Gene Ontology</td>
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<tr>
<td>GSH</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>HDF</td>
<td>Human dermal Fibroblast</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>JNK</td>
<td>Jun nuclear kinase</td>
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<td>Abbreviation</td>
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<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia gene and genomes</td>
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<td>L</td>
<td>Litre</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
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<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
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<td>Ml</td>
<td>millilitre</td>
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<td>Mm</td>
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<tr>
<td>MMP-1</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NF-kB</td>
<td>Necrosis Factoe kappa B</td>
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<tr>
<td>NGN</td>
<td>NaringeniN</td>
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<tr>
<td>°C</td>
<td>Degree celsius</td>
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<tr>
<td>PUFA</td>
<td>Poly unsaturated fatty acid</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNA-seq</td>
<td>Ribonucleic acid sequencing</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>TE</td>
<td>Trolox equivalent</td>
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<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1

INTRODUCTION

Good skin integrity is vital to a healthy life. Skin health and beauty is considered one of the major factors representing overall well-being. The aging process is a challenging human experience common to everyone, and the desire to look young prevails in the majority of the global population. With the elongation of life span and quality of life of global population, skin appearance becomes extremely important for people to look attractive and boost confidence in their social interactions. In today’s world, there is a great quest for eternal youth and an insatiable desire for methods which could reverse the biological clock. This has led to the willingness to spend fortune on cosmetics products, and has resulted in numerous cosmetic products flooding the market with no detail studies. Anti-aging products are mainly supplements and cosmeceutical skin care products with hope of creating a younger consumer look by lowering, prevention, and masking of skin aging signs. These signs include laxity (sagging), rhytids (wrinkles), photoaging effects such as solar elastosis, erythema, keratosis, dyspigmentation and poor texture. Anti-aging treatments may also focus on particular agent of skin aging for example exposure to sunlight (Asma et al., 2014).

The craving of man to remain perpetually young is dated back to 4000 B.C., evidences were obtained from archaeological artefacts used for cosmetics purposes found in Egypt, and these were believed to be an integral part of Egyptian dressing (Britannica, 2015). The cosmetics industry is classified as one of the world's fastest growing industries, it's considered to be a necessity instead of things people want for materialistic purpose (Eze et al., 2012). The Industry’s revenue in 2012 was estimated to be $245 billion (€180 billion) and $292 billion for 2015. Americans spend nearly $7 billion a year on cosmetics and $10.1 billion on cosmetics procedures (http://rosiemolinary.com/2011/10/23/the-price-of-beauty/retrieved 19th July 2016). A report on global anti-aging market trend by Transparency Market Research (TMR, 2014) estimated anti-aging market segment from the total cosmetics market to worth USD $191.7 billion globally by 2019 with an annual growth rate of 7.8% between 2013–2019 (TMR Analysis, 2014). According to the report, two regions that carry maximum potential in this segment over the forecast period are Asia – Pacific and Rest of World (RoW). India, China, Japan, and South Korea are poised to attract maximum interest in for anti-aging or age – reversing procedures further supported by the respective government of these Countries (TMR Analysis, 2014). The market value for the Asia – Pacific has increased to more than USD $70 billion, which is the second largest market after western European market.

The Malaysia's cosmetics industry has been witnessing tremendous growth, market size has been growing and consumer demand is rising spontaneously. In few years 40% in growth was witnessed from MYR 1.4 billion Malaysia ringgit in 1995 to MYR 1.9 billion in 2007 (Swidi et al., 2010) projecting total sales value to hit USD $1.1 billion by 2010 with 13% annual growth rate. According to the Malaysia department
Small, medium and large cosmetics company are developing wide range of anti-aging ingredients but desirable features of anti-aging agents such as efficacy, affordable price, safety and mechanisms of action are still lacking. Despite the huge spending and time invested in rejuvenation procedures, most products found on the shelves of cosmetics stores either lacked the proven efficacy claimed by the manufacturer or possess side effects detrimental to human’s health (Avantaggiato et al., 2015; Grosicki, et al., 2014; McEwen, et al., 2012; Miyamura et al., 2011; Zouboulis & Makrantonaki 2011; Odumosu & Ekwe, 2010). Also, there are numerous drawbacks cause by prolong application of some of the synthetic anti-aging cosmetics products such as skin cancer, irritations, inflammations, DNA damage (Suresh, 2014); endocrine disruption and skin absorption of toxic chemicals such as 2, 4 – D, DEET etc. (Pont, et al., 2004; Sarveiya, 2004; Charron & Brand, 2004). Chemical compounds such as hydroquinone, homosalate, paraben which either serve as Sunscreen or whitening agents are found in Anti-aging cosmetics products and reports have shown that these compounds are toxic to the skin. (Makrantonaki & Zouboulis 2012; Brand, et al., 2003). As a consequence of the negative side effects, anti-aging researchers are now looking into the use of natural products as alternative solution to slowing down the skin aging process (Divya et al., 2015; Mohamed et al., 2014; Pérez-Sánchez et al., 2014; Fujii et al., 2013; Hasham et al., 2013; Chiang et al., 2011; Choi et al., 2010; Park et al., 2010; Angerhofer et al., 2009; Kim et al., 2009). Therefore, there is need to explore the scientific validity on herbs usage as anti-wrinkle, and their activity should be further explore (Mukherjee et al., 2011).

In this project, 10 Zingiberaceae rhizomes from Malaysian traditional plants which are used in folkloric practices for the management of skin wrinkles were selected for this studies. They are Zingiber zerumbet, Curcuma xanthorrhiza, Boesenbergia rotunda, Kaempferia galanga, Curcuma mangga, Curcuma aeroginosa, Zingiber officinale var. rubrum, Curcuma longa, Alpina cochigera and Zingiber officinale.

Thus, the hypothesis of this study states that extract from one sample of Zingiberaceae sp. with high antioxidative capacity will induced the up regulation and down regulation of genes or transcription factors related to aging in cellular model of UV induce skin aging.

The general objective of this study is to determine the anti-aging properties of Zingiberaceae using normal human adult dermal fibroblast cells as a model while the specific objectives of this study are:

1. To screen 10 Zingiberaceae plant species for their Antioxidative properties.
2. To determine, quantify specific flavonoid and profile the biochemical compositions in the selected Zingiberaceae samples.
3. To evaluate the effects of *Zingiberaceae* extracts on the inhibition of extracellular degrading enzymes (ECM).

4. To evaluate the proliferative effects and *in vivo* toxicity assessment of *C. xanthorrhiza* and *C. longa* using normal human adult dermal fibroblast cells and Zebrafish (*Danio rerio*) as a model.

5. To identify the differential expressed gene (DEG), annotate genes and identify major pathways involved in both UV-irradiated and UV-Irradiated normal human dermal fibroblast *C. xanthorrhiza* extract treated.

6. To validate gene expression profile using quantitative polymerase chain reaction (qPCR).
REFERENCES


